Antibody-Based Strategies for Identifying Novel Apoptotic-Cell Surface-Associated Molecules

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Abstract

The recognition and phagocytosis of apoptotic cells is not only a physiological means of waste disposal but can also result in anti-inflammatory and immunosuppressive effects. Indeed, defective clearance of apoptotic cells (ACs) is associated with autoimmune and inflammatory disease states such as systemic lupus erythematosus. To date, relatively few markers have been identified as 'eat me' signals displayed on AC surfaces despite the great potential for such molecules as diagnostic or therapeutic reagents. In this work various antibody-based strategies were employed in an attempt to identify novel AC-specific epitopes. An initial strategy utilised a phage displayed antibody library containing a repertoire of $\sim 10^8$ antibody fragments encoded by human germline genes as an unbiased source of binding specificity. An alternative approach was based on the knowledge that receptors used by macrophages to recognise ACs also recognise pathogen-associated molecules. By looking for the ability of antibodies raised against pathogens to cross-react with ACs the hypothesis that cells undergoing apoptosis reveal molecular patterns that resemble those on pathogen related structures was tested. Screening of phage-displayed antibodies isolated from *in vitro* selections performed on both whole ACs and on preparations of purified AC-associated molecules did not identify any AC-specific epitopes. However, screening of antibodies raised *in vivo*, that have previously been characterised as having specificity for pathogen-associated molecular patterns (PAMPs) revealed that some cross-react with cells undergoing apoptosis. One of these antibodies was found to bind an epitope found on the ubiquitously expressed ~40KDa precursor to Laminin-Binding-Protein (LBP/p40). These findings suggest that epitopes resembling PAMPs appear on the surface of

mammalian cells as a result of apoptosis and that these epitopes can be found on endogenously expressed molecules which are normally excluded from the surface of viable cells. The ability of host receptors to cross-react with host and pathogen-associated epitopes in this way may lead to a greater understanding of the progression of inflammatory, infectious and tumoural processes.

Declaration

This is to certify that: -

(i) *the thesis comprises only my original work towards the PhD.*

(ii) *the thesis is less than 100,000 words in length, exclusive of tables, bibliographies and appendices.*

IAN TENNANT

15th November, 2004

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Abbreviations

2%MPBS	2% Skimmed milk powder in PBS
3M K+/5M Ac-	Potassium acetate buffer
Ab	Antibody
ABCA1	ATP-binding cassette transporter-1
ACAMP	Apoptotic-cell-associated molecular pattern
AmpR	Ampicillin resistance
Apaf1	Apoptosis activating factor-1
APS	Ammonium persulphate
ASGP-R	Asialoglycoprotein-receptor
ATP	Adenosine triphosphate
AxV	Annexin V
β_2GPI	Beta 2-glycoprotein I
β_2GPI-R	Beta 2-glycoprotein I -receptor
BCL-2	B cell lymphoma/leukaemia gene-2
BL	Burkitts' lymphoma
bp	Base pairs
BSA	Bovine serum albumin
CD	Cell differentiation
CED	Programmed cell death deficient
CHO	Chinese hamster overy
CHX	Cycloheximide
CR	Complement receptor
CRP	C-reactive protein
CsCl	Caesium Chloride
DAPI	4',6-diamidino-2-phenylindole,dihydrochloride
DC	Dentritic cell
dH_2O	Distilled water
DIABLO	Direct IAP-binding protein with low pI
DLVO	Derjagin-Landau/Verwey-Overbeek
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECL	Electrochemical luminescence
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbance assay
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Gas-6	Growth-arrest specific gene 6
GFP	Green fluorescent protein
GPI	Glycosyl-phosphatidylinositol
GTE	Glucose-Tris-EDTA buffer
HBS	Hepes buffered saline
HCl	Hydrochloric acid
HEK	Human embryonic kidney
HEPES	N-[2-hydroxyethyl] piperazine-N' -[2-ethanesulfionic acid]
HMDM	Human monocyte-derived macrophage
HRP	Horse radish peroxidase
IAP	Inhibitors of apoptosis

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-2	Interleukin-2
IMDM	Iscoves modified Dulbecco's medium
KCl	Potassium chloride
kDa	Kilodaltons
Kdo	a2-keto-D-mammo-3-deoxyoctulosonic acid
LB	Lauria Bertani broth
LBP	LPS-binding protein
LBP/p40	Laminin-binding Protein
LDL	Low-density lipoprotein
LOX1	Lectin-like oxLDL receptor
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALDI-TOF-MS	Matrix-assisted laser desorption-ionization/ time-of-flight-mass spectormetry
MBL	Mannose binding lectin
MeOH	Methanol
MFG-E8	Milk fat globule-EGF-factor 8
$MgCl_2$	Magnesium chloride
Muc-1	Mucin-1
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEDD5	Neuronally-expressed developmentally-downregulated-5
NGS	Normal goat serum
OFA	Onco-fetal antigen
OPD	O-phenylenediamine
oxPC	Oxidised phosphatidylcholine
oxPL	Oxidised phospholipids
oxLDL	Oxidised low-density lipoprotein
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% v/v Tween 20
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Propidium Iodide
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
PS	Phosphatidylserine
PSR	Phosphatidylserine receptor
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
RBC	Red blodd cell
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SAP	Serum amyloid protein
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrlimide gel electrophoresis
SEM	Standard error of means
SHPS-1	Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1

siRNA	Small interfering RNA
SLE	Systemic lupus erythmatosus
Smac	Second mitochondria-derived activator of caspase
SPA	Surfactant protein A
SPD	Surfactant protein D
SRA	Scavenger receptors class A
SR-B1	Scavenger receptors class B1
sst	Somatostatin receptor
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
TEMED	N,N,N'N'-tetramethylenediamine
TetR	Tetracycline resistance
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumour-necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Tris	Tris(hydroxymethyl)-aminomethane
Triton-X-100	Polyethylene glycol tert-octylphenyl ether, t-Octylphenoxypolyethoxyethanol, 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol
Tween 20	polyoxyethylene sorbitan monolaurate
UV	Ultraviolet

1 Introduction

1.1 Requirements for timely clearance

From observations made *in vivo*, the existing paradigm followed by those who study programmed cell clearance is that apoptotic cells undergo changes at their cell surface that act as "eat-me signals" and that engulfment by phagocytes, the culmination of the apoptosis process, occurs early during this programme such that the membrane integrity of the dying cell is maintained prior to engulfment. These beliefs arise from and are supported by the sparseness of apoptotic bodies in histological sections of healthy tissue (Kerr, Wyllie et al. 1972). Furthermore, if one follows the development of the nematode, *Caenorhabditis elegans*, an organism that lacks macrophages, the so-called "professional phagocytes", swift engulfment of cells that are pre-programmed to die can be observed before they show visible evidence of apoptosis (Sulston and Horvitz 1977; Ellis, Jacobson et al. 1991). Under this school of thought, the work described in this thesis aims to identify novel apoptotic cell-associated "eat-me signals". Accordingly, the literature reviewed in the following introduction will describe not only the current understanding of apoptotic cell-associated ligands and engulfment receptors identified to date but will also attempt to highlight examples where this paradigm fails to operate for *in vitro* investigation of dying-cell engulfment. For example, why are long incubation periods and huge excesses of apoptotic cells required for engulfment by macrophages *in vitro*?

1.2 Perspectives on apoptosis

1.2.1 A gardener's perspective

The roles fulfilled by apoptosis in tending to a well-kept body can be likened to the tasks carried out by a gardener managing his plot. Programmed cell death (PCD) by apoptosis trims and prunes organs during the development and growth of organisms in order to create and maintain a defined cellular and tissue landscape. In contrast to the unplanned necrotic havoc caused by violent changes to a cell's local climate, the process of apoptosis conscientiously ensures a cell's membrane-bagged refuse is "flagged" in time for collection by garbage-disposing phagocytes. Thus littering of the surrounding environment is avoided. The ligation of a cell's death receptors, "weeds" out transformed or pathogen-infected cells that would otherwise overwhelm the stable grounds of a tissue.

1.2.2 An historical perspective

Naturally occurring cell death has been noted in publications dating back to the nineteenth century. The observations of cell death in the notochord of metamorphic toads by Carl Vogt (Vogt 1842)* were followed by discoveries that included the massive cell death that occurs in pupating diptera by Weismann (Weismann 1864)*. Several years passed until phagocytosis associated with cell death was described by Metchnikoff (1883) in the muscles of metamorphic toads (Metchnikoff 1883)* and chromatolytic death, akin to "apoptotic" cell death, in ovarian follicles recorded by Flemming in 1885 (Flemming 1885)* (* cited in Clarke and Clarke 1996). Later, whilst carrying out experiments to investigate "absorption of the formed elements"

Metchnikoff noted, in great detail, the phagocytosis of goose "red corpuscles" that had been injected into a number of different invertebrates and vertebrates (Metchnikoff 1905). The attributes of this form of cell death continued to be recorded with only the changing of name from karyorrhexis or pyknosis until the formation of the "apoptosis concept" in 1972, by Kerr et al. (Kerr, Wyllie et al. 1972). Since then many components involved in the apoptosis process have been cloned and characterised, and studies have provided an appreciation of not only the components of pathways controlling initiation and execution of the these events (Hengartner 2000) but have led to the understanding of a functional signalling module linking the apoptotic cell to the phagocyte (Akakura, Singh et al. 2004).

1.2.3 A therapeutic perspective

All steps in the apoptosis programme have been linked to disease - from the abortion of apoptosis as a mechanism for resistance to cancer therapy (Miyashita and Reed 1992) to roles for the caspases following stroke (Loddick, MacKenzie et al. 1996) and in heart disease (Buja 1998). Increased apoptosis in the absence of inflammation is a suggested means for the progression of fibrosis (Henson 2003), whereas an abundance of apoptosis with defective clearance can lead to autoimmune disease (Herrmann, Voll et al. 1998).

Theoretically, there are a number of ways in which apoptosis can (and has) been implicated in loss of self-tolerance and hence autoimmune disease. Defective apoptosis can lead to a failure to silence or eliminate autoreactive T- or B- cells (McDonnell, Deane et al. 1989), but it is an excess of apoptosis or failure to clear apoptotic cells due to compromised phagocytic mechanisms that is central to the interest of the work described in this thesis.

Studies of the serological abnormalities in systemic lupus erythmatosus (SLE) have provided important evidence for the mechanisms linking defective clearance and autoimmune disease. That is, the association of deficiencies in complement components and the body's ability to clear dying self (Taylor, Carugati et al. 2000), and the presence of autoantibodies, which can not only react with apoptotic cells, but also can induce apoptosis, augmenting tissue damage and potentially leading to antibody Fc-mediated inflammation (Nakamura, Shidara et al. 1994). Indeed, mounting evidence suggests that SLE autoantigens are derived from apoptotic cells (Cocca, Seal et al. 2001; Coleman, Sahai et al. 2001; Cocca, Cline et al. 2002). Therefore, understanding the molecular interactions operating in apoptotic-cell recognition will allow the prevention or restoration of many diseased states. However the identification of numerous SLE-disease-susceptibility genes (such as those regulating threshold for tolerance and induction and those modifying the appearance of SLE in specific organs (Mason and Isenberg 1998)), in addition to those required for antigen clearance raises the question of whether defective clearance is the causative factor, or whether it serves only to amplify the adaptive immune response to self antigens.

Animal models offer an ideal means to query the importance of clearance genes in disease progression (Taylor, Carugati et al. 2000). However, despite the great potential of these *in vivo* model systems, there is a risk of inbred strains providing a "primed" background upon which the effects of deficiency in the target gene can be amplified over and above that which would occur on a normal genetic background (Botto, Dell'Agnola et al. 1998; Gillmore, Hutchinson et al. 2004). Such a pitfall has been exposed recently through studies performed by Botto and colleagues, who have

identified a susceptibility locus, derived from 129 and C57BL/6 mice, that is sufficient to mediate the loss of tolerance to nuclear antigens. This phenotype, which, in many cases had previously been attributed to disrupted "clearance-genes", demonstrates important epistatic modifiers of autoimmunity in this widely used strain, and suggests that these background gene influences may account for some, or even all, of the autoimmune traits described in some gene-targeted models of SLE (Bygrave, Rose et al. 2004)

1.2.4 A scientist's perspective

1.2.4.1 The choreography and morphology

Although a variety of stimuli can induce apoptosis, two main pathways, that is an "extrinsic" and an "intrinsic" pathway (see figure 1-1), function to produce the morphological traits associated with this form of cell death. Given an appropriate death signal, a conserved and ordered sequence of activities activated post-translationally from resident molecules leads to irreversible death commitment through the triggering of several essential elements. The best characterised of these is a proteolytic cascade of cysteine-dependent aspartate-specific proteases, or caspases (Alnemri, Livingston et al. 1996). Once activated these cytosolic enzymes cleave specific substrates, resulting in the ordered dismantling of a cell and giving rise to the morphological changes that are characteristic of apoptosis.

Proteins of the Bcl-2 family also play an important role in deciding whether a cell commits itself to die. Members of this family can exert either pro- or anti- apoptotic outcomes within a cell. The functional attributes of a particular protein relate to the structural domains that it possesses, with the anti-apoptotic Bcl-2 proteins having

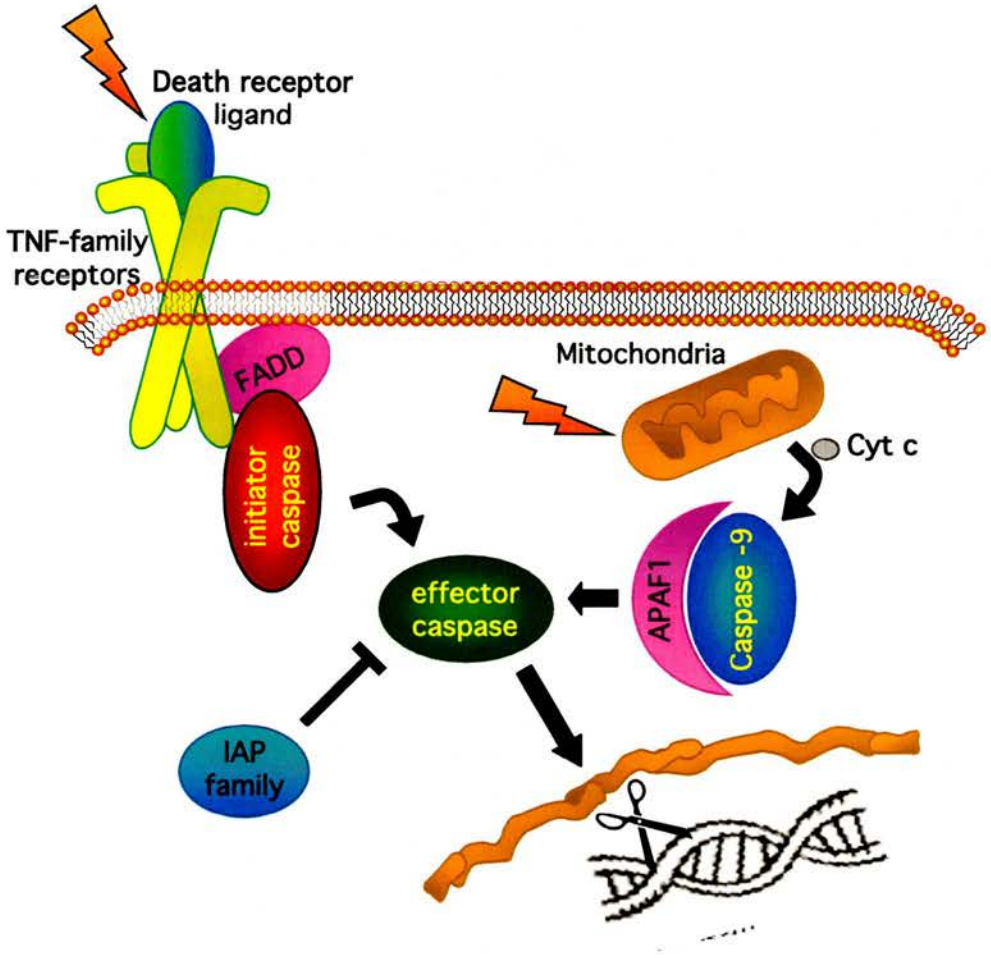


Figure 1-1. The two main pathways that lead to apoptosis.
The **extrinsic pathway** begins outside a cell, when conditions in the extracellular environment determine that a cell must die. Ligation of a death receptor (such Fas (CD95) with Fas ligand (FasL or CD95L)) results in clustering of receptors and initiates the extrinsic pathway. Clustering of the TNF-family receptors ("death receptors") recruits FADD and pro-initiator-caspases (such as pro-caspase 8) to the complex. Concentration of pro-initiator caspases results in its autocatalysis and activation; activated initiator-caspases cleaves pro-effector-caspases (such as pro-caspase 3), which then undergoes autocatalysis to form active effector-caspases. The **intrinsic apoptosis** pathway begins when a cell detect stress or injury. Mitochondrial damage, for example, can initiate the intrinsic pathway, overcoming the effect of the pro-survival protein Bcl2 (not shown). Cytochrome c (cyt c), released from damaged mitochondria, binds Apaf1, which then activates an initiator-caspase, (such as caspase 9), which then activates an effector-caspase (such as caspase 3). Other proteins released from damaged mitochondria, such as Smac/DIABLO, counteract the effect of IAPs (inhibitor of apoptosis proteins), which normally bind and prevent activation of caspase 3. The balance between Bcl-2 family members and proteins such as, IAPs, and Smac/DIABLO is central to the intrinsic apoptosis pathway.
Both the extrinsic and intrinsic pathways merge at effector-caspases, such as caspase 3, which then cleave various proteins including endonucleases leading to DNA degradation.

four Bcl-2 homology (BH) domains (BH 1-4) and a transmembrane anchor sequence, whereas the pro-apoptotic proteins lack, at the very least, a BH4 domain, but must possess a BH3 domain (Adams and Cory 1998). Through the ability of many Bcl-2 members to form heterodimers as well as homodimers, the overall result of their presence seems to rest upon the absolute level of either pro- or anti-apoptotic members. Thus cells with more pro-apoptotic proteins allow the release or activation of downstream pro-apoptotic factors, such as cytochrome c, from mitochondrial stores into the cytosol (Yang, Liu et al. 1997). Several hours are usually required from the initiation of cell-death to the final cellular disintegration, although the exact timing depends on the cell type, the stimulus, and the apoptotic pathway. Nevertheless, when viewed microscopically, the morphological consequences of these events are easily followed. Early during the initiation of apoptosis, cells lose contact with neighbouring cells. Following this, extensions of plasma membrane, commonly known as blebs can be seen to form, the cells shrink and the blebs separate, forming apoptotic bodies. The characteristic morphological changes seen in the nucleus of apoptotic cells are condensation of chromatin starting at the periphery along the nuclear membrane, leading to the formation of a ring-like structure before fragmentation.

The end result of this process is the phagocytic removal of the dying cells. *In vivo* this clearance serves the purpose of eliminating functionally inappropriate cells without eliciting either a pathological or inflammatory response (Wyllie, Kerr et al. 1980). Evolutionary conservation of genes both for initiating and executing apoptosis, and for engulfing corpses is indicative of the value of this process (Bangs,

Franc et al. 2000; Gumienny and Hengartner 2001; Tosello-Trampont, Brugnera et al. 2001; Grimsley, Kinchen et al. 2004).

1.2.4.2 Putting it in motion - attachment and phagocytosis

Phagocytosis is an evolutionarily conserved process employed by eukaryotic cells to ingest both microbial pathogens, and components of "dying-self". Recent studies have shown that both cytoskeletal alterations and membrane trafficking are necessary for the phagocytic event to occur (reviewed in (Greenberg and Grinstein 2002)). Many molecules controlling the signal transduction pathways leading to cytoskeletal alterations required for engulfment have been cloned and characterised (table 1-1). However, before phagocytosis can occur, receptors must recognise ligands on the approaching particle in order to establish firm adherence (figure 1-2).

Whilst biologists focus on characterising specific receptor and ligand molecules, physicists have developed theoretical and experimental methods for relating the interaction energy between the approaching surfaces to their properties. So, in physical terms adherence cannot occur until an electrostatic repulsion between the negatively charged surface of a phagocyte and target cell has been overcome. From the Deryagin–Landau/Verwey–Overbeek (DLVO) theory of colloid physics, the energy of interaction of two charged particles of like sign and magnitude is the sum of the electrostatic repulsion and the energy of attraction provided by electromagnetic interactions due to London-Van der Waal's forces. Although this theory does not account for the fine molecular details found on a cell-surface, penetration through the electrostatic repulsive barriers (such as those imparted by a charged glycocalyx) will be minimised by a small radius of curvature between the approaching surfaces. For this reason, it has been proposed that initial cell contact is

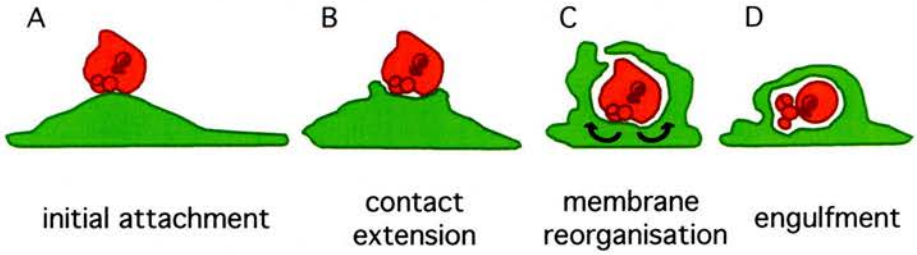


Figure 1-2. Sequential phases of attachment and phagocytosis. The stages of attachment and engulfment of an apoptotic cell by a phagocyte are depicted A-D. A: Contact first involves a limited area. B: "Contact extension" requires cell flattening at the micrometer level for efficient cross-linking of interacting surfaces by receptors (Pierres, Benoliel et al. 2002) to prevent disruption of non-covalent bonds mediating adhesion. C: Engagement of signaling receptors allows morphological changes to occur, a result of cytoskeletal and membrane reorganisation (Gumienny, Brugnera et al. 2001)(Ogden, deCathehneau et al. 2001). D: finally the cell corpse is enclosed within the phagocyte and phagosome fusion with endosomes results in digestion.

	Caenorhabditis elegans	*Drosophila melonigaster*	Mammalian	Reference
Receptors	CED 1	Draper	LRP	Freeman, Delrow et al. 2003 Zhou, Hartwieg et al. 2001
	CED 7		ABCA1	Wu and Horvitz 1998
		Crq	CD36	Franc, White et al. 1999
	F29B9.4	dPSR	PSR	Fadok, Bratton et al. 2000
Signal transducers	CED 6		GULP	Liu and Hengartner 1998
	CED 2		CrkII	Reddien and Horvitz 2000
	CED 5		DOCK 180	Wu and Horvitz 1998
	CED 12		ELMO	Gumienny, Brugnera et al. 2001
	CED 10		Rac	Reddien and Horvitz 2000

Table 1-1. Evolutionarily conserved genes involved in apoptotic cell clearance. Abbreviations: - ELMO; engulfment and motility. GULP; engulfment and adaptor protein. LRP; CD91/low density lipoprotein receptor-related protein. PSR; Phosphatidylserine recep

promoted, in the case of a macrophage, by its rough surface (Mege, Capo et al. 1987).

The study of factors influencing bacterial attachment to phagocytes often includes characterisation of the gross differences in surface properties such as charge and hydrophobicity which allow preferential interaction of specific bacterial species (Dickson and Koohmaraie 1989) (Briandet, Meylheuc et al. 1999). Several influential studies in the area of apoptotic-cell clearance also explored such differences; these often led to the identification of interactions centred on specific receptors and ligands. Examples of such studies include the role of neutrophil surface-charge in determining recognition by macrophages (Savill and Haslett 1987) (Savill, Henson et al. 1989) and the measurement of microelectrophoretic mobility by Morris *et al* who found that the surface charge-density of apoptotic cells was less than that of non-apoptotic cells (Morris, Hargreaves et al. 1984).

Through extension of their earliest studies Savill and Haslett identified the CD36/vitronectin receptor/thrombospondin receptor complex as an apoptotic-cell recognition system. Similarly, the demonstration that thymocyte recognition by macrophages is inhibited by N,N'-diacetyl chitobiose and N-acetyl glucosamine (Duvall, Wyllie et al. 1985) led to the discovery of a role for the asialoglycoprotein receptor (ASGP-R) in this process. The ASGP-R, which is expressed on the plasma membrane of hepatocytes, detects exposed galactose residues of asialated glycoproteins allowing endocytosis of asialoglycoprotein complexes for recycling by the liver cells. As well as being involved in the general removal of aged proteins, the ASGP-R provides a classic example of a multifunctional protein that enables the engulfment of senescent self by bystander cells (Dini, Autuori et al. 1992). Thus, it

seems that through the process of evolution, phagocytes have obtained sets of receptors to allow them to recognise specific targets. However, macrophages also adhere to glass and plastic and can ingest inert particles such as latex beads, yet the likelihood that specific cell-surface receptors for these ligands exist is slim. In remembering the early studies by Savill and Haslett or Wyllie and colleagues, it is interesting to speculate whether "non-specific" forces influence interactions between apoptotic cells and macrophages, in modes that have been demonstrated for other cellular attachment systems.

Thus, whilst acknowledging the importance of specific interactions between cell surface receptors and their ligands for firm adhesion, Bongrand and colleagues have also shown the influence of nonspecific forces in mediating the kinetics of initial cellular attachment (Bongrand 1998). For example, in these studies the equilibrium shape of a cell (an important factor for attachment) adhering to a surface was found to be a balance between freely diffusible cell surface receptors and repulsion generated by bulky surface structures composing the glycocaylx (see figure 1-3) (Bell, Dembo et al. 1984). Furthermore, it has been suggested that numerous "nonspecific" interactions of wide-ranging affinities between a variety of surface molecules may play a role in the adhesion when specific adhesion receptors display low density affinity or accessibility due to their size (Pierres, Benoliel et al. 2002).

However, in the case of bacterial attachment to host surfaces it has been argued that the same basic, physiochemical forces are responsible for so-called 'non-specific' and 'specific' binding and that from a physiochemical point of view the distinction between the two is an artificial one (Busscher, Cowan et al. 1992). So let us now

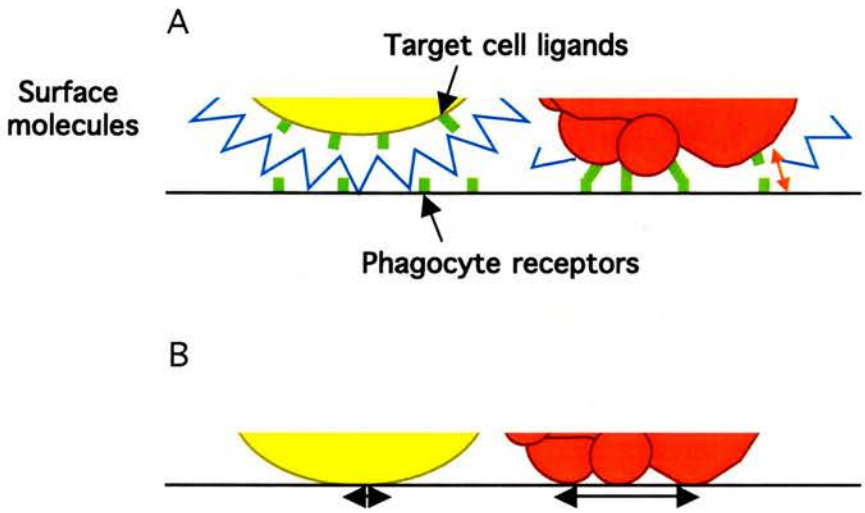


Figure 1-3. Possible mechanisms for an increased adherence of apoptotic cells.

A As the equilibrium shape of a cell adhering to a surface is a balance between freely diffusible cell surface receptors and repulsion generated by bulky surface structures (Bell, Dembo et al. 1984) alterations in membrane fluidity (i.e ABCA1(Hamon, Chambenoit et al. 2002)) and shedding of surface molecules (Hart, Ross et al. 2000) during programmed cell death may increase adherence through improved accessibility. Alternatively, in the case of short range interactions at the margin of contact region (red arrow) increased diffusion of steric repellant cell-surface molecules on an apoptotic cell might allow greater contact extension during initial interaction than allowed for viable cell surfaces.

B The deformed surface of an apoptotic cell may also provide an extended area of contact with phagocyte membranes perhaps enhanced by ligand receptor interactions leading to additional membrane deformations (absence of signaling through CD31(Brown, Heinisch et al. 2002)).

consider the existing knowledge regarding such specific sites on the apoptotic-cell surface.

1.2.5 A macrophage's perspective

1.2.5.1 "Fit for consumption"- a target cell's requirements

In comparison to chaotic changes associated with cell death by necrosis, PCD is an organised process (Kerr, Wyllie et al. 1972). In keeping with this, an apoptotic cell must engage efficient mechanisms to take on a guise that is different from its viable form in order to appear edible to an engulfing cell. To date there is no firm evidence of either the direct products of the proteolytic cascade that occurs during apoptosis, or *de-novo* synthetic products serving as ligands for recognition and phagocytosis apoptotic cells. Table 1-2 lists the apoptotic cell-associated ligands for engulfment which have been identified thus far along with implicated cognate receptors, where known. These are generated as a result of either (a) changes to cell-surface resident molecules or (b) arise from a redistribution of intracellular molecules to the cell surface. Examples of each will be reviewed separately in the following sections.

1.2.5.2 Recognition and repulsion signals previously implicated on the surface of apoptotic cells

1.2.5.2.1 Changes to cell-surface resident molecules

1.2.5.2.1.1 Intercellular Adhesion Molecule-3 (ICAM-3)

The discovery that ICAM-3 may be involved in the clearance of apoptotic cells was made following the observation that certain monoclonal antibodies to leucocyte

surface molecules block the ability of human-monocyte-derived macrophages (HMDM) to recognise apoptotic lymphocytes *in vitro* (Flora and Gregory 1994). Although being expressed at high levels on the surface of both viable lymphocytes and macrophages (de Fougerolles and Springer 1992), subsequent studies found this particular feature of ICAM-3 to be exclusive to the apoptotic-cell associated molecule as opposed to the macrophage (Moffatt, Devitt et al. 1999).

Despite much structural and functional knowledge (looking at both molecular interactions, and the consequence of signalling) of receptor-ligand interactions involving ICAM-3 on viable leucocytes (Vilella, Mila et al. 1990; Cid, Esparza et al. 1994; Landis, McDowall et al. 1994; Bell, May et al. 1998; Serrador, Vicente-Manzanares et al. 2002), the details of how ICAM-3 mediates macrophage interaction with apoptotic cells remains a mystery (Shingler 2003). The hypothetical ICAM-3-associated epitope found solely on apoptotic cells (i.e. an "apoptotic" form of ICAM-3) is one of the main targets for the phage antibody studies described in this thesis (results chapters 1 and 2). Thus, in the following sections, whilst reviewing the mechanisms by which cell-surface resident molecules are known to become altered during apoptosis, where appropriate, proposals will be made as to how the same mechanisms could mediate alterations in the ICAM-3 found on dying cells.

Cell surface receptors	Bridging molecule	Apoptotic cell ligand	Reference
FcγR	SAP	PE, chromatin, DNA	Mold, Baca et al. 2002. Familian, Zwart et al. 2001
$\alpha_v\beta_{3-5}$	milk fat globule-EGF-factor 8	PS, PE	Hanayama, Tanaka et al. 2002. Akakura, Singh et al. 2004
PSR	-	PS	Fadok, Voelker et al. 1992. Fadok, Bratton et al. 2000
β_2GPI-R	$\beta_2$glycoprotein	PS	Balasubramanian, Chandra et al. 1997
c-Mer	GAS6	PS	Scott, McMahon et al. 2001. Nakano, Ishimoto et al. 1997
MER/Tyro-3	Protein S	PS	Anderson, Maylock et al. 2003
?	annexin V	PS	Tait, Gibson et al. 1989
	IgM	PC	Shaw, Horkko et al. 2000
FcγR	CRP	oxPL	Chang, Binder et al. 2002
SHPS-1	-	CD47	Tada, Tanaka et al. 2003
ASGP-R	-	Modified carbohydrate residues	Dini, Autuori et al. 1992. Dini, Lentini et al. 1995
Lectins ?	-	Modified carbohydrate residues	Duvall, Wyllie et al. 1985. Rapoport, Khaidukov et al. 2003)
?	-	ICAM-3	Moffatt, Devitt et al. 1999
?	-	Annexin I	Arur, Uche et al. 2003
Annexin I/II	-	PS	Fan, Krahling et al. 2004
CD31	-	disabled CD31	Brown, Heinisch et al. 2002
ABCA1	-	ABCA1 (PS?)	Luciani and Chimini 1996
CD14	?	?	Devitt, Moffatt et al. 1998
CD91/Calreticulin	C1q	?	Korb and Ahearn 1997. Ogden, deCathelineau et al. 2001
CD91	SP-A, SP-D	?	Vandivier, Ogden et al. 2002
SRA/Calreticulin	MBL	?	Nauta, Raaschou-Jensen et al. 2003
CD36/$\alpha_v\beta_{3-5}$	Thrombospondin-1	?/PS	Savill, Hogg et al. 1992
CD36/β_2	-	?/PS	Godson, Mitchell et al. 2000
SRA	-	PS	Platt, Suzuki et al. 1996
SR-B1	-	oxidised-LDL-like sites ?	Fukasawa, Adachi et al. 1996
CD68	-	oxidised-LDL-like sites ?	Ramprasad, Fischer et al. 1995
LOX-1	-	oxidised-LDL-like sites ?	Oka, Sawamura et al. 1998
Complement receptors	(i)C3, C4	require complement activation	Mevorach, Mascarenhas et al. 1998. Morelli, Larregina et al. 2003

Table 1-2. Receptors and ligands implicated in the recognition and clearance of apoptotic cells. Abbreviations: - ABCA1; ATP-binding cassette transporter-1. ASGP-R; Asialoglycoprotein-receptor. β2GPI; Beta 2-glycoprotein I. β2GPI-R; Beta 2-glycoprotein I –receptor. Gas-6; Growth-arrest specific gene 6. LOX1; Lectin-like oxLDL receptor. MBL; Mannose binding lectin. OxPC; Oxidised phosphatidylcholine. OxPL; oxidised phospholipids. PE; Phosphatidylethanolamine. PSR; Phosphatidylserine receptor. SAP; Serum amyloid protein. SHPS-1; Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1. SPA; Surfactant protein A. SPD; Surfactant protein D. SRA; Scavenger receptors class A. SR-B1; Scavenger receptors class B1. ?; uknown/unconfirmed.

1.2.5.2.1.2 Oxidation-specific epitopes

Recent studies have demonstrated that the generation of free radical intermediates and subsequent oxidative stress may be more than unavoidable events of the apoptotic machinery and that these by-products may act as signals for the execution of the apoptosis programme and subsequent recognition of apoptotic cells. Early observations showed that oxidised LDL (OxLDL) can compete with apoptotic cells undergoing increased oxidative stress for binding by macrophages (Sambrano and Steinberg 1995). This led to the demonstration that oxidatively modified moieties on the surfaces of apoptotic cells serve as ligands for macrophage recognition in the same way as similar moieties on oxidized low-density lipoprotein (OxLDL) (Chang, Bergmark et al. 1999). Subsequent studies have shown that some oxidized phospholipids bind to lysine residues of apolipoprotein B100 (apoB100) to provide ligands for macrophage scavenger receptors (Gillotte, Horkko et al. 2000).

Although it will be discussed further in section 1.2.5.2.2.1.1, where the best-known feature of the surface of apoptotic cells, the exposed phosphatidylserine (PS) will be considered in detail, it is worth mentioning here that the generation of oxidised PS from PS during apoptosis may be requisite for its recognition (Kagan, Borisenko et al. 2003). However, in addition to the formation oxidised PS Podrez *et al.* have identified a family of oxidised phosphatidylcholine (PC) homologues that can also serve as ligands for the apoptotic-cell receptor CD36 (Podrez, Poliakov et al. 2002). Yet, as will be discussed in section 1.2.6 when looking into receptors that may recognise these changes, the occurrence of these specific events on the surface of cells may be a relatively late event of apoptosis.

1.2.5.2.1.3 Redistribution and clustering

With changes in membrane fluidity known to occur during programmed cell death (Jourd'heuil, Aspinall et al. 1996) there is great scope for the types of redistribution of surface molecules that have been observed in other biological scenarios, such as the removal of erythrocytes from the circulation following oxidation-induced aggregation of membrane glycoproteins (i.e. band 3) (Beppu, Takahashi et al. 1994). Evidence that apoptotic cells can transiently cluster surface molecules at an early stage of apoptosis to provide novel sites for recognition by macrophages has been provided by studies involving CD43 (Eda, Yamanaka et al. 2004). In the system used by Eda *et al.*, Jurkat cells treated with etoposide or anti-Fas antibody transiently became susceptible to binding and phagocytosis by THP-1 cell-derived macrophages even before displaying chromatin condensation but in a caspase-dependent manner. Further studies indicated that the sialylpolylactosaminyl sugar chains of CD43 might provide multivalent-high affinity ligands for recognition by macrophage receptors. A similar type of redistribution during apoptosis may also occur with ICAM-3, as redistribution of the glycoprotein to uropods (protrusions at the posterior pole of cells) has been observed in polarised migrating T lymphocytes (del Pozo, Nieto et al. 1998).

1.2.5.2.1.4 Altered glycosylation

Due to their complexity and diversity, carbohydrate units can convey a great deal of information and effect important functions in biological systems. Carbohydrate-containing molecules mediate many types of cell-to-cell interactions; L-selectin on naïve T-cells will only bind the correctly glycosylated form of CD34 (i.e. only CD34

molecules containing sulfated carbohydrates) and promote the rolling interactions critical to the selectivity of naïve lymphocyte homing (Satomaa, Renkonen et al. 2002). A combination of clustering and differential glycosylation has recently been held responsible for a process akin to apoptotic-cell engulfment, that is the clearance of transfused platelets from the circulation (Hoffmeister, Josefsson et al. 2003). Hoffmeister *et al* have shown that cooling of blood platelets clusters the von Willebrand factor receptor complex and have suggested that the macrophage integrin $\alpha_M\beta_2$ recognises exposed N-acetylglucosamine residues of N-linked glycans of this complex. Enzymatic galactosylation of chilled platelets was shown to block $\alpha_M\beta_2$ recognition thus prolonging the circulation of functional cooled platelets. The early studies by Dini *et al.* eluded to the possible appearance of differentially glycosylated residues on apoptotic cells (Dini, Autuori et al. 1992; Dini, Lentini et al. 1995). Rapoport and colleagues who have identified a glycosylation patterns specific to apoptotic cells have highlighted such a possibility recently. In their system, monocyte-derived THP-1 cells were shown to engulf the apoptotic bodies from the MELJUSO melanoma cell line that show a carbohydrate pattern consisting of high levels of glycans terminated by galactose or sialic acid. The inhibition of binding by oligosaccharide-specific reagents was observed in this system along with evidence of binding to siglec and galectin ligands by monocyte-derived THP-1 cell (Rapoport, Khaidukov et al. 2003). Together these findings led to the conclusion that the Galβ1 - 3GalNAcβ-terminated chains displayed on the apoptotic bodies appeared to be targets for THP-1 cells.

Changes in glycosylation pattern have also been suggested as a means by which apoptotic-cell associated ICAM-3 is able participate in clearance (Moffatt, Devitt et

al. 1999). ICAM-3 is expressed as a type 1 transmembrane protein with five extracellular Ig domains. Up to two thirds of its molecular weight (110-130kD in T-lymphocytes and 120-160kD in neutrophils) results from N-linked glycosylation (Fawcett, Holness et al. 1992; de Fougerolles, Diamond et al. 1995). Deglycosylated ICAM-3 can still support binding by LFA-1 (Landis, McDowall et al. 1994) demonstrating that the carbohydrate moieties are not a prerequisite for its ligand function with this receptor, though the carbohydrates may be important in mediating its interaction with dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN) (VanderVieren, LeTrong et al. 1995) (Geijtenbeek, Torensma et al. 1999). As a member of the C-type lectin family, it is capable of binding ICAM-3 with high affinity in a Ca^{2+} -dependent, mannan/mannose inhibitable manner (Geijtenbeek, Torensma et al. 2000). Given that small amounts of the high mannose-type oligosaccharides were detected during oligosaccharide analysis on ICAM-3(Funatsu, Sato et al. 2001), it seems plausible that these could provide the epitope for recognition by DC-SIGN or a similar phagocyte receptor for "apoptotic ICAM-3".

1.2.5.2.1.5 Active detachment

The idea that "eat me" signals (Savill, Fadok et al. 1993) appear on the apoptotic-cell-surface satisfactorily explains how a dying cell may become desirable to a phagocyte. Nevertheless, the immunoglobulin superfamily members, ICAM-3 and CD31, which are recognised by phagocytes for the purpose of apoptotic-cell engulfment (Moffatt, Devitt et al. 1999; Brown, Heinisch et al. 2002), can also mediate adhesive interactions between viable cells that do not lead to engulfment. As has been suggested for ICAM-3 (section 1.2.5.2.1.1), subtle changes to such molecules may allow a phagocyte to discriminate between "viable" and "apoptotic"

forms. Yet monoclonal antibodies to epitopes on ICAM-3 and CD31 that inhibit apoptotic cell clearance also bind to the same molecules on viable cells. An alternative explanation as to molecules implicated in clearance merely serving as "eat-me" signals was provided by the studies of CD31 by Brown and colleagues.

1.2.5.2.1.5.1 CD31

CD31 can bind several different partners and function as, amongst other things, an intercellular adhesion-stabilising molecule on viable endothelial cells to stabilise intercellular junctions (Newman, Berndt et al. 1990). Through homophilic engagement, CD31 also functions as an intracellular signal-triggering molecule, mediating the migration of leucocytes across endothelia (reviewed in (Jackson 2003)) and the active repulsion of viable leucocytes from macrophages (Brown, Heinisch et al. 2002). Brown and colleagues proposed that by losing the ability to transmit "detachment" signals during apoptosis, CD31 promotes tethering of dying cells to phagocytes.

1.2.5.2.1.6 Conformation and homophilic/heterophilic association

Despite providing compelling evidence of a detachment signal, the means by which CD31 loses its function remains undefined. A change in conformation of the extracellular domain of this and other immunoglobulin superfamily members (such as ICAM-3) could well lead to an alteration in their functioning. Indeed Johnson and colleagues have suggested such a mechanism for similar cell-adhesion molecules. Using direct force measurements to investigate how Ig domains contribute to the adhesive interactions of the neural-cell-adhesion molecule (NCAM), as a representative of this protein class of adhesion molecules Johnson and colleagues

demonstrated that NCAM binds in two spatially distinct configurations. Following studies of various Ig-domain deletion mutants it was suggested that a combination of multiple bound states and internal molecular flexibility allows synergistic bond formation and the ability to accommodate different intercellular spaces (Johnson, Fujimoto et al. 2004).

Together, these examples illustrate that the surface of an apoptotic cell is sensitive to factors that may generate novel epitopes for recognition. However many of these observations originate from studies *in vitro* where a degree of cytolysis is constitutively present within cultures. This is likely to cause release of enzymes and reactive oxygen species to the extracellular milieu modifying surface molecules to an extent that may not occur *in vitro*.

1.2.5.2.2 Redistribution of intracellular molecules to the cell-surface

It appears that apoptosis allows the exposure of molecules that are usually forbidden from leaving the intracellular environment of viable cells. Given that the innate immune system recognises microbial structures both by virtue of their dissimilarity to host structures, and through the existence of conserved molecular patterns, it is enticing to think that recognition by pattern-recognition-receptors (PRRs, see also section 1.2.6) is through common determinants that exist on both microbial and normally "hidden" intracellular-self derived host molecules.

1.2.5.2.2.1 Anionic phospholipids and loss of membrane asymmetry

In its resting state in viable cells, the plasma membrane retains aminophospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner

leaflet of the plasma membrane, whereas choline-containing phospholipids such as phosphatidylcholine (PC) and sphingomyelin are found mostly in the outer leaflet. The asymmetric distribution of phospholipids is achieved by an ATP-dependent aminophospholipid translocase that specifically transports aminophospholipids from the outer to the inner leaflet. Relocation from the inner-leaflet is a result of decreased aminophospholipid translocase activity and activation of a calcium-dependent lipid scramblase (Tilley, Cribier et al. 1986; Bevers, Comfurius et al. 1999).

1.1.3.2.2.1.1 Phosphatidylserine

The most well-known surface change common to the vast majority apoptotic cells is the appearance of phosphatidylserine on the outer-leaflet of the plasma membrane (Fadok, Voelker et al. 1992). Fadok and colleagues eluded to the possibility that the recognition and removal of the apoptotic cell by phagocytes is a direct consequence of this change after observing the stereospecific inhibition of apoptotic-cell phagocytosis by phosphatidylserine and its structural analogues, but not by other anionic phospholipids.

1.1.3.2.2.1.1.1 PS is PS is PS

Although there is no doubt that many phagocytic receptors bind PS, there is growing evidence that changes to phosphatidylserine in addition to its externalisation are required for the engulfment of target cells (Sambrano, Terpstra et al. 1997). Oxidative stress is a well-known component of apoptotic signalling (section 1.2.5.2.1.2). By labelling cellular phospholipids with an oxidation-sensitive, fluorescent fatty acid (*cis*-parinaric acid) it has been possible to characterise

phospholipid oxidation during oxidative stress-induced apoptosis (Hedley and Chow 1992). These studies revealed that preferential oxidation of PS as a result of apoptosis leads to its externalisation (Arroyo, Modriansky et al. 2002). Furthermore, inhibition of PS oxidation in cells during apoptosis has been demonstrated to block phagocytosis of dying cells by macrophages (Kagan, Gleiss et al. 2002). As a consequence of these findings it has been proposed that a combination of PS and oxidised PS may be essential for recognition and uptake of apoptotic cells (Kagan, Borisenko et al. 2003), although the exact amounts of oxidised PS actually present or required on the cell surface to facilitate recognition of apoptotic cells is unknown.

1.2.5.2.2.1.2 Phosphatidylethanolamine

The early studies of Fadok *et al.* emphasised the importance of PS in preference to other anionic phospholipids for apoptotic-cell recognition. Several molecules since implicated in the process also appear to bind to PE. Human serum amyloid P component (SAP), a glycoprotein structurally belonging to the pentraxin family of proteins (section 1.2.6.2.2), was originally noted as having a role in controlling the degradation of chromatin following the observation that mice lacking SAP develop antinuclear antibodies (Bickerstaff, Botto et al. 1999) and autoimmunity (see also section 1.2.6.1.5 for discussion on strain dependency) (Gillmore, Hutchinson et al. 2004). However, SAP also binds to apoptotic cells via exposed PE, an interaction which is strongest on late, permeable apoptotic cells (Familian, Zwart et al. 2001). Binding to PE is also likely to be a significant contributing factor to the affinity of the opsonin milk fat globule-EGF-factor 8 (MFG-E8) to apoptotic cells (Hanayama, Tanaka et al. 2002)

1.2.5.2.3 Translocation of cellular compartments

In 1984 Wyllie and colleagues noted both small irregular blebs of cytoplasm and deep surface pits during their electron microscopic analysis of apoptotic thymocytes (Morris, Hargreaves et al. 1984). Since then, the former of these morphological changes has received much more attention than the latter. Such surface protrusions, or blebs, have been shown to contain fragmented endoplasmic reticulum (ER) and ribosomal components as well as nucleosomal DNA and ribonucleoproteins and have been held as the proposed site for exposure of autoantigens (Casciola-Rosen, Anhalt et al. 1994). However, it was the surface pits that Wyllie and colleagues suggested to be caused by accelerated fusion of membranes from endoplasmic reticulum. Recently Lowe *et al.* have observed the very type of impaired membrane trafficking during apoptosis that could cause such events (Lane, Lucocq et al. 2002) (Lowe, Lane et al. 2004). They have shown caspase-dependent cleavage of the Golgi-associated transport factors giantin and syntaxin 5, which is accompanied by a cessation of vesicular transport between the ER and the Golgi complex, and first manifests itself as a block in ER exit (Lowe, Lane et al. 2004). They propose that such interdomain cleavage of fusion and tethering factors would not only inhibit normal trafficking pathways but may allow "inappropriate" fusion reactions to occur (Lowe, Lane et al. 2004). Perhaps surface expressed ER or Golgi-derived molecules from the apoptotic cell could provide ligands compatible with the molecules from the endoplasmic reticulum on the side of the macrophage, a source of membrane that has been shown to be used for phagosome formation (Gagnon, Duclos et al. 2002). However, many of these phenomena appear late during apoptosis (personal communication- Jon Lane) and a function in apoptotic-cell clearance has yet to be

ascribed to them. On the other hand, following the discovery that Annexin I is exposed on the cell surface following translocation from the cytosol during apoptosis, Arur *et al.* have shown its colocalisation with phosphatidylserine on surface blebs and, in the nematode *Caenorhabditis elegans*, downregulation of an annexin I homologue prevents efficient engulfment of dying cells (Arur, Uche et al. 2003). More recent work has shown that annexin 1 can be presented on the surface of apoptotic cells prior to the externalisation of PS (Macasev, Weyd et al. 2004)

Although not being studied with respect to functioning as a ligand for engulfment, a similar type of exposure of another intracellular protein, actin, has been noted on the surface of apoptotic cancer cells prior to loss of membrane integrity and demonstrates that this phenomenon is a property of cytoskeletal components (Hansen, Nielsen et al. 2001). These studies demonstrated that actin can be recognised by tumour-infiltrating B cells in the context of a dying cell's surface.

These findings are not confined to proteins originating from the cytoplasm as was demonstrated originally following the discovery that human anti-DNA autoantibodies can bind to blebs of apoptotic Jurkat cells (Cocca, Seal et al. 2001). This interaction was found to be dependent on the activity of both caspases and ROCK I kinase, a stimulator of nuclear fragmentation and distribution and bleb formation (Cocca, Cline et al. 2002).

1.2.5.2.4 Chemotactic factors

The clearance of dying cells that takes place in the interdigital zones during development is a classical example of macrophage accumulation at sites of apoptotic lesions (Saunders 1966). Recent *in vitro* studies have reinforced the long-held suspicion that apoptotic cells draw macrophages to sites of cell-death by releasing

chemotactic factors. Following the detection of a chemoattractant activity for primary human macrophages in the supernatants of apoptotic cells, Lauber *et al.* identified lysophosphatidylcholine, which is released from dying cells as a factor responsible for this activity (Lauber, Bohn et al. 2003). Thrombospondin too, has also been shown to actively recruit macrophages when released from apoptotic fibroblasts (Moodley, Rigby et al. 2003). Although not explicitly being demonstrated as chemoattractants for mature macrophages, apoptotic cell-derived blebs and cross-linked homodimers of S19 ribosomal proteins attract monocytes suggesting that dying cells may release a cocktail of factors to draw macrophages to the sites of an apoptotic lesion *in vivo* (Horino, Nishiura et al. 1998; Segundo, Medina et al. 1999). Through observing *C. elegans* mutants of CED-5 or CED-12 the importance of the same cellular adjustments required to effect engulfment has also been realised for the process of migration (Wu and Horvitz 1998) (Gumienny, Brugnera et al. 2001) (Grimsley, Kinchen et al. 2004).

1.2.6 Engulfment receptors and mechanisms

Unearthing the receptors used by phagocytes for the recognition and clearance of apoptotic cells has revealed remarkable parallels between the receptors used by the innate immune system to detect pathogens. These include both membrane-bound receptors and soluble opsonins or binding-proteins that bridge apoptotic cells with phagocytes. In their role as microbial receptors, these molecules were collectively referred to as "pattern recognition receptors" (PRRs) (Medzhitov and Janeway 2000), due to their ability to recognise conserved molecular patterns shared by large groups of microorganisms, allowing the innate immune system to discriminate self from the pathogen-associated molecular patterns (PAMPs) found on non-self

(Janeway 1989). It has also been proposed that the use of PRRs for recognition of effete self is due to the presence of apoptotic-cell-associated molecular patterns (ACAMPs) by these cells (Franc, White et al. 1999).

At this point it is useful to define what is understood by a "molecular pattern" in this context. Any molecule can be identified by a three dimensional arrangement of chemical elements which gives rise to a unique structural "fingerprint". Therefore microbes will have countless "molecular patterns" in addition to the pathogen-associated-molecular-patterns (PAMPS) customarily referred to in the context of immunological recognition. The structural basis by which single PRRs recognise both "molecular patterns" on microbes (PAMPs) and apoptotic cells (ACAMPs) is not known, that is whether the PRR recognises the same pattern present on different molecules or whether it has evolved different sites capable of recognising different patterns. The ability of these receptors to interact with such a broad range of ligands (Aderem and Ulevitch 2000) (Jack 2000) (exemplified below in the case of CD14 section 1.2.6.1.1) has been attributed to the presence of a specific configuration of ionic charge arising from a particular complementary "molecular pattern" on a molecule which determines if it may serve as a ligand for a given receptor.

Table 1-2 shows a summary of membrane receptors and bridging molecules identified along with their associated ligands on the apoptotic-cell-surface, where known. Depending on the ligand some of these can participate in the clearance of apoptotic cells that are at an early stage of programmed cell death (for example by the PSR following the appearance of PS, section 1.2.6.1.5) whereas for others interaction appears to be during later stages of death (for example by C-reactive protein following loss of membrane integrity, section 1.2.6.2).

1.2.6.1 Membrane bound receptors

1.2.6.1.1 CD14

CD14 was first implicated in the clearance of apoptotic cells during the screening of mAbs to leucocyte antigens for the ability to inhibit the recognition of apoptotic cells by human monocyte-derived macrophages. Monoclonal antibody 61D3 was identified as a blocking antibody (Flora and Gregory 1994). However, several years passed before the epitope recognised by 61D3 was successfully identified as CD14 (Devitt, Moffatt et al. 1998), a 55kDa glycosylphosphatidylinositol (GPI)-linked plasma membrane glycoprotein of mature myeloid cells or a soluble serum glycoprotein lacking the GPI-linked anchor (Bazil, Baudys et al. 1989; Simmons, Tan et al. 1989).

1.2.6.1.1.1 *CD14 Ligands*

Together, numerous studies have suggested that CD14 has an affinity not only for microbial-associated molecules of various species but also for molecules associated with self. Table 1-3 is a summary listing these ligands. In considering these ligands, it should be appreciated that classification of individual molecules as ligands for CD14, has, in many cases, been based on signalling responses merely dependent on the presence of the receptor, without demonstrating a direct interaction with the "ligand" being studied. Thus, in many cases the absence of contaminants capable of inducing a CD14-dependent response, such as LPS, has not been confirmed.

1.2.6.1.1.1.1 Lipopolysaccharide

The first functional role assigned to CD14 was as a cellular receptor for lipopolysaccharide (LPS) that enables mediation of LPS-induced pro-inflammatory responses (Wright, Ramos et al. 1990). The definitive mechanisms of LPS induced signalling through CD14 are unclear but the most widely accepted model is that CD14 in some way facilitates the delivery of LPS to Toll-like receptor-4 (TLR-4) (Qureshi, Lariviere et al. 1999) promoting its dimerisation with the assistance of MD-2 (Shimazu, Akashi et al. 1999) to form a signalling complex containing myeloid differentiation factor 88 (MyD88) leading to activation of the MAP kinase and NF-κB-inducing cascades. (reviewed Beutler, Hoebe et al. 2003). The binding of LPS to CD14 is enhanced by the LPS-binding protein (LBP), a 60kDa acute phase serum glycoprotein, (Tobias, Soldau et al. 1986; Schumann, Leong et al. 1990) however the potential for LBP to enhance apoptotic-cell recognition has not been investigated.

1.2.6.1.1.1.1.1 LPS structure

Structural characterisation of many LPSs has shown them generally to conform to a structure, shown schematically in figure 1-4, which can be divided into three separate regions. Lipid A is the hydrophobic and endotoxically active part of the molecule (Tanamoto, Galanos et al. 1984). Covalently attached to this is the core section that can itself be divided into an inner and outer core. The inner core, nearest to lipid A, contains a high proportion of unusual sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep). The outer core extends further from the bacterial surface and is more likely to consist of common sugars such as

hexoses and hexoseamines. Onto this is attached, in most cases, a polymer of repeating subunits called *O*-polysaccharide, or *O*-chain, also typically composed of hexoses. However the *O*-chain is often truncated giving rise to lipo-oligosaccharide (LOS) in certain rough strains of bacteria (Erridge, Bennett-Guerrero et al. 2002).

1.2.6.1.1.2 CD14-dependent clearance of apoptotic cells.

Even though numerous eukaryotic host-associated ligands for CD14 have been proposed (shown in table 1-3) the component(s) of apoptotic cells which CD14 engages during their engulfment remains unspecified. Observation that CD14-dependent clearance of apoptotic cells operates in both human and murine systems using feed cells of a number of different lineages (Devitt, Moffatt et al. 1998; Schlegel, Krahling et al. 1999; Devitt, Pierce et al. 2003) (Fadok, Konowal et al. 1998) suggests that the ACAMP(s) recognised by CD14 are relatively conserved structure(s). As a classical pattern recognition receptor (PRR), CD14 appears to recognise numerous, often seemingly unrelated molecules in addition to LPS, both from microbes and non-microbial origin that can be lipid, protein or carbohydrate (table 1-3). As transient transfection of CD14 in COS cells conferred increased phagocytic ability to these cells it is unlikely that CD14 requires other macrophage-specific receptors to function in apoptotic-cell clearance (Devitt, Moffatt et al. 1998). These studies also provided evidence that CD14 acts in a separate pathway from the $\alpha_V\beta_3$/TSP/CD36 complex and more recent work has shown that, although CD14 can bind phospholipids of eukaryotic cells including PS, it does not function preferentially as a PS-receptor in apoptotic-cell clearance (Devitt, Pierce et al. 2003). A link between the ICAM-3 dependent clearance pathway and CD14 pathway was originally suggested by the work of Moffat et al (Moffatt, Devitt et al. 1999).

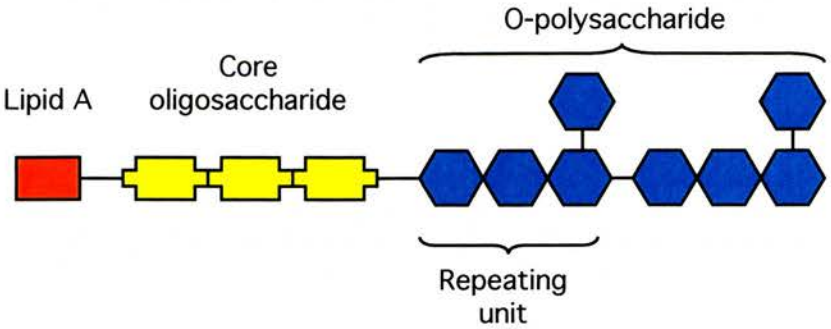


Figure 1-4. Schematic representation of the structure of Lipospolysaccharide (LPS)

Ligand	Reference
Bacterial	
Lipopolysaccharide	Wright, Ramos et al. 1990
Cell wall components (not LPS)	Fichorova, Cronin et al. 2002
Rhamnose glucose polymers	Soell, Lett et al. 1995
Uronic acid polymers	Espevik, Otterlei et al. 1993. Jahr, Ryan et al. 1997
Teichuronic acids	Yang, Sugawara et al. 2001
Lipotechoic acid	Schwandner, Dziarski et al. 1999
Peptidoglycan	Weidemann, Schletter et al. 1997
Synthetic muramyl dipeptide	Weidemann, Schletter et al. 1997
Lipoarabinomannan	Pugin, Kravchenko et al. 1998
Flavolipin	Gomi, Kawasaki et al. 2002
Glycolipids	Schroder, Opitz et al. 2000
Soluble Tuberculosis factor (STF)	Medvedev, Henneke et al. 2001
Outer membrane lipoproteins	Wooten, Morrison et al. 1998
Acylpolygalactoside	Hmama, Mey et al. 1994
Group B *Streptococcus* (released soluble factor)	Henneke, Takeuchi et al. 2001
Components of *Staphylococcus aureus*	Kusunoki, Hailman et al. 1995
Components of gram positive organisms	Pugin, Heumann et al. 1994
Other prokaryotic/viral	
WI-I Yeast cell wall protein	Newman, Chaturvedi et al. 1995
RSV fusion (F) protein	Kurt-Jones, Popova et al. 2000
Hyphal fragments	Wang, Warris et al. 2001
Fucoidan	Heinzelmann, Polk et al. 1998
Non-microbial	
Taxol	Perera, Vogel et al. 1997
Chitosans	Otterlei, Varum et al. 1994
Lipopetides	Sellati, Bouis et al. 1998
Interleukin-2	Bosco, Espinoza-Delgado et al. 1997
Heat Shock proteins	Kol, Lichtman et al. 2000
Phospholipids	Wang, Kitchens et al. 1998
Ceramide	Pfeiffer, Bottcher et al. 2001
Surfactant-proteins A and D	Sano, Chiba et al. 2000
Lactoferrin	Baveye, Elass et al. 1999
Minimally modified low density lipoproteins	Miller, Viriyakosol et al. 2003

Table 1-3. Ligands of CD14. Many of these molecules have been classified as ligands for CD14 based on signalling responses in the presence of the receptor, without demonstrating a direct receptor ligand interaction. That is, the absence of contaminants capable of inducing a CD14 dependant cellular activation, such as LPS, has not been confirmed.

However, recent studies, although not excluding this possibility have failed to demonstrate a direct interaction between these two molecules (Shingler 2003). Despite the fact that not all CD14 ligands share gross structural features, one may propose that CD14 recognises conserved patterns of ionic charge representing parts of microbial and self molecules (including exposed ACAMPs) (Ulmer, Dziarski et al. 1999). In making this assumption, however, one may then ask why the response to apoptotic cell engulfment is anti-inflammatory (Fadok, Bratton et al. 1998; McDonald, Fadok et al. 1999) whereas that of CD14 dependent LPS recognition is pro-inflammatory. An answer to this may lie in the interaction of these ligands with receptors subsequent to tethering by CD14. For example, evidence suggests that signalling through toll-like receptors (TLRs), despite being critical for maturation of microbial containing phagosomes, is not implicated in apoptotic cell clearance (Blander and Medzhitov 2004) (Cvetanovic and Ucker 2004).

1.2.6.1.2 Scavenger receptors

The scavenger receptor (SR) group consist of a broad range of transmembrane molecules containing, amongst others, collagenous, cysteine-rich and C-type-lectin domains (Brown and Goldstein 1990; Kodama, Freeman et al. 1990). SRs that have been implicated in the uptake of apoptotic cells are SR-A, SR-B1, lectin-like oxidised LDL-receptor 1 (LOX-1), CD36 and CD68 (oxLDL receptor or macrosialin) (Savill, Hogg et al. 1992; Ramprasad, Fischer et al. 1995; Fukasawa, Adachi et al. 1996; Platt, Suzuki et al. 1996; Oka, Sawamura et al. 1998). They can be found not only on professional phagocytes such as macrophages and dendritic cells but also differentially expressed on some endothelial, cerebral and retinal cells, where they are involved either directly or indirectly in the binding and phagocytosis

of unwanted self, such as oxLDL and apoptotic cells in addition to many classes of microbial pathogens and their products. They provide an excellent example of tissue specific-usage with respect to apoptotic-cell clearance as cells of immune privileged sites such as Mato cells (granular epithelial cells of neural tissue), astrocytes, cerebral microvascular endothelial cells, and retinal pigment epithelial cells selectively employ one or more of these receptors for the engulfment of dying self (Mato, Ookawara et al. 1996; Husemann, Loike et al. 2002).

CD36, one of the first engulfment receptors to be implicated in the recognition of apoptotic cells, was formerly studied with respect to platelet aggregation, as a receptor for thrombospondin (Asch, Barnwell et al. 1987). A role in apoptotic-cell clearance was demonstrated by anti-CD36 antibody blockade and through observing conferred ability to non-phagocytic cells to engulf apoptotic cells following CD36 gene transfer (Savill, Hogg et al. 1992; Ren, Silverstein et al. 1995). CD36 appears to work in association with the vitronectin receptor ($\alpha_V\beta_3$ integrin, CD51/CD61) to bind the 420 kDa adhesive glycoprotein, thrombospondin, which itself forms a bridge by binding to unknown sites on the apoptotic cell. (Savill, Hogg et al. 1992). Studies have also shown CD36 to be a necessary cofactor in PS-mediated recognition of apoptotic cells in some systems (Fadok, Warner et al. 1998).

The importance of SRs for the recognition of apoptotic cells is seen through their usage by *Drosophila melanogaster* and *C. elegans*. The haemocytes of *Drosophila melanogaster* use Croquemort (Crq), a homologue of the mammalian CD36 to remove cells undergoing apoptosis during embryogenesis (Franc, Dimarcq et al. 1996). The *C. elegans* homologue to CD91 (class-F scavenger receptor from endothelial cells (SREC)) (Zhou, Hartwieg et al. 2001) (Su, Nakada-Tsukui et al.

2002) was originally identified by failure of cells in mutants of CED-1 protein to cluster around cell corpses in mutants also defective in the engulfment gene ced-7 (see also table 1-1).

The general lack of insight into the structural basis for apoptotic-cell recognition is emphasised by studies examining the epitope recognised by the classical receptor for oxLDL, CD68, as it has been shown that lipid micro-emulsions prepared from oxLDL can inhibit the binding of both intact oxLDL and apoB from oxLDL supporting the conclusions that at least some of the macrophage receptors for oxidized LDL can recognize both the lipid and the protein moieties (Bird, Gillotte et al. 1999).

1.2.6.1.3 Calreticulin

Originally identified as an intracellular calcium-binding protein, calreticulin also functions as a chaperone for proteins synthesised in the endoplamic reticulum (Fliegel, Burns et al. 1989; Wada, Imai et al. 1995). Furthermore, calreticulin can also be found at the cell surface (White, Zhu et al. 1995), and it is here that the molecule appears to be involved in the clearance of dying self through it's association with CD91 and engagement of C1q, MBL, SPA or SPD (Ogden, deCathelineau et al. 2001; Vandivier, Ogden et al. 2002). On the other hand, when released from necrotic cells, calreticulin is recognised by SR-A in prefernce to CD91 (Berwin, Hart et al. 2003).

1.2.6.1.4 Lectins

As mentioned in section 1.2.4.2 early studies into apoptotic-cell clearance suggested involvement of macrophage lectins (Duvall, Wyllie et al. 1985) (Morris, Hargreaves

et al. 1984). In addition to a role for the asialoglycoprotein receptor (Dini, Autuori et al. 1992), it has been shown that increased cell surface expression of mannose receptors in the liver can also enhance the recognition of apoptotic lymphocytes (Dini 2000). However aside from the soluble binding proteins that display lectin-like properties (section 1.2.6.2.1) other candidates for membrane bound receptors mediating these events include CD68 and CD14 both of which display lectin-like properties (Holness and Simmons 1993; Weidemann, Schletter et al. 1997).

1.2.6.1.5 The phosphatidylserine receptor

Many membrane receptors and indeed opsonins (see table 1-2) involved in apoptotic cell clearance can bind to PS, although only the recently described phosphatidylserine receptor (PSR) appears to be specific for this phospholipid. Originally discovered as an activity expressed on macrophages stimulated with digestible particles such as beta-glucan (Fadok, Laszlo et al. 1993) Fadok *et al* went on to clone the PSR (Fadok, Bratton et al. 2000) as a protein expressed on the surface of macrophages, fibroblasts and epithelial cells and which when transfected into B- and T-lymphocytes, enables them to recognise and engulf apoptotic cells in a phosphatidylserine-specific manner. Prior to the discovery of the PSR, several other candidates were proposed, including CD14, an oxLDL receptor (CD68), CD36, annexins, beta 2 glycoprotein I, milk fat globule-EGF-factor 8, and gas-6. Although studies suggest that CD14 does not appear to contribute to PS dependent apoptotic-cell recognition (Devitt, Pierce et al. 2003), it is possible that these candidates (suggested recently for annexins I and II (Fan, Krahling et al. 2004)) may recognise PS *in vivo*.

Observations that psr-1, the Caenorhabditis elegans homologue of the PSR, is important for cell corpse engulfment and that *in vitro* psr-1 binds preferentially to phosphatidylserine or cells with exposed phosphatidylserine (Wang, Wu et al. 2003), along with evidence that in PSR-deficient mice, dead cells accumulated in the lung and brain, causing abnormal development (hyperplasic brain phenotype) and neonatal lethality (Li, Sarkisian et al. 2003) was initially interpreted as proof for a universal role for the receptor in corpse clearance during development. Conversely, recent data, which also demonstrated perinatal lethality, growth retardation and a delay in terminal differentiation of the kidney in PSR knockout mice, found engulfment of apoptotic cells to be normal. However, PSR-deficient macrophages were impaired in pro- and anti-inflammatory cytokine signalling after stimulation with apoptotic cells or with lipopolysaccharide, suggesting that the PSR may be essential for the development of organs but not for apoptotic cell removal (Bose, Gruber et al. 2004). These latter disclosures are in keeping with earlier reports that the PSR can localise to the nucleus through multiple nuclear localisation signals (Cui, Qin et al. 2004).

Observation that overexpression of "the PSR" confers a phagocytic capacity that is sterospecifically inhibited by PS (Fadok, Bratton et al. 2000) is in keeping with initial studies which provided unequivocal evidence for the existence of a receptor capable of sterospecifically recognising PS on the surface of apoptotic cells (Fadok, Voelker et al. 1992). However, whether the molecule, currently refered to as "the PSR", plays a physiologically relevant role in apoptotic-cell clearance remains to be confirmed or refuted.

1.2.6.1.6 ATP binding cassette transporter (ABCA1)

ABCA1 (or ABC1), the first of a subclass of 12 ABC transporters which is involved in the outward redistribution of PS at the plasma membrane (Hamon, Broccardo et al. 2000) has been implicated in programmed cell-clearance on both the side of the macrophage and the apoptotic cell. Evidence for a role of phagocyte-associated ABCA1 in engulfment was provided by the impaired ability of peritoneal macrophages to engulf apoptotic thymocytes following antibody blockade of the molecule (Luciani and Chimini 1996) and the close structural resemblance to the nematode engulfment gene *ced-7* (Moynault, Luciani et al. 1998; Wu and Horvitz 1998). Although no formal demonstration of direct transport of PS by ABCA1 has been reported it has been proposed that this molecule favours engulfment via the generation of lipid-determined directional forces sufficient to promote membrane sprouting around the particle to be ingested (Hamon, Chambenoit et al. 2002).

1.2.6.2 Opsonins

In addition to direct engagement of apoptotic cells by membrane bound receptors, a number of soluble opsonins have been shown to bridge apoptotic cells with phagocytes. Whilst many of these molecules, such as β2 glycoprotein I, milk fat globule-EGF-factor 8, and gas-6 have been shown to bind PS (section 1.2.6.1.5), the binding-site for other opsonins, such as thrombospondin (section 1.2.4.2) is unknown. Members of the collectin and pentraxin family, and complement factors, which have been implicated in apoptotic cell clearance will be discussed in more detail below.

1.2.6.2.1 Complement components and collectins

C1q, the initiating factor of the classical pathway of complement activation binds directly to apoptotic cells via its globular head domain (Korb and Ahearn 1997). This event may also induce complement activation with subsequent deposition of C4b and C3b (Nauta, Trouw et al. 2002) which can lead to an immunosuppressive switch following internalisation by dendritic cells via complement receptors 3 and 4 (Mevorach, Mascarenhas et al. 1998; Morelli, Larregina et al. 2003). In addition, C1q has been shown to link apoptotic cells to phagocytes via the binding of natural antibodies or mannose binding lectin (MBL) (Ogden, deCathelineau et al. 2001). Structurally, C1q resembles the collectin family of proteins although it shares no sequence homology with these C-type lectins. Examples of other members of this family implicated in apoptotic-cell clearance include the lung surfactant protein (SP) A, SP-D and C-reactive protein (CRP) (Schagat, Wofford et al. 2001) (Chang, Binder et al. 2002; Vandivier, Ogden et al. 2002). In the case of CRP, the epitope recognised on the surface of dying cells has been partially characterised as oxidized phosphatidylcholine, but for other opsonins, such as C1q, MBL, SP-A, and pentraxin-3 (PTX3), the ligand on the apoptotic-cell surface is currently unknown.

1.2.6.2.2 Pentraxins

Members of this protein family are characterised by their pentameric organisation and include the glycoprotein serum amyloid P component (SAP). Mice which lack the SAP gene develop autoantibodies to nuclear antigens, which was originally interpreted as evidence for a role of SAP in controlling the degradation of chromatin (Bickerstaff, Botto et al. 1999). However, as SAP was also known to bind to

phosphatidylethanolamine (section 1.2.5.2.2.1.2) Familian *et al* performed studies to demonstrate that this specificity allows SAP to opsonise apoptotic cells independently of its nuclear binding. Calcium-dependent binding of SAP was observed in early, nonpermeable apoptotic Jurkat, SKW, and Raji cells (Familian, Zwart et al. 2001).

The role of opsonins in the uptake of early apoptotic cells remains to be established as data suggest that C1q, MBL and the pentraxins are primarily important in the clearance of apoptotic cells in later stages of the cell death process, after they have become leaky (Nauta, Raaschou-Jensen et al. 2003) (Nauta, Trouw et al. 2002). Therefore, in the normal clearance of early apoptotic cells, other clearance mechanisms must operate, involving recognition of molecules which can detect early changes. Nevertheless, the contribution of C1q to the clearance system is apparently important as C1q-deficient mice show impaired clearance of apoptotic cells, and since both C1q-deficient mice and humans are strongly susceptible to autoimmune disease (Botto, Dell'Agnola et al. 1998).

Although dogma states that free late apoptotic cells rarely occur *in vivo*, there is the possibility that in situations when the load of apoptotic cells exceeds the local available phagocytic ability cells will proceed to late stages of apoptotic death (often referred to as necrosis secondary to apoptosis) prior to clearance, for example, following incidents of infection or inflammation. Such observations have been made through attempts to experimentally induce inflammation *in vivo* (Medan, Wang et al. 2002; Devitt, Parker et al. 2004)

Therefore systems may exist to manage clearance in either case (Fujii, Shiratsuchi et al. 2001; Wiegand, Corbach et al. 2001; Shiratsuchi, Mori et al. 2003). As the

induction of apoptosis *in vitro* results in a concoction of cells at early and late stages of death it is not easy to know exactly what stage a particular cell is at when the phagocyte or purified receptor meets it. What is more, a cell may progress to later stages of death following "assessment of apoptosis" during treatment for assays (see results chapter 3).

It is not surprising that for a process so physiologically important as apoptotic-cell clearance, evolution has designed multiple receptors capable of recognising a multiple apoptotic-cell associated ligands to be used for both tethering and signalling for engulfment of the dying cell. Collectively, the organised multi-protein interactions involved in connecting a corpse to an engulfing cell has been termed a "phagocytic synapse" (Savill, Dransfield et al. 2002; Ravichandran 2003)

Proposals as to why so many receptor/ligand interactions are involved include redundancy or that different sets of receptors are used for ligands as they appear on a cell during different phases of apoptosis. Alternatively it could be that that the presence of multiple low-affinity receptors allows for sensitivity during the initial binding stage, such that just one or two molecules initiate tethering followed by a more complicated union during engulfment. This is the case for molecules involved in T-cell:APC interactions which characteristically have weak affinities, (in the low micromolar range) but yet with apparent specificity (Davis, Krogsgaard et al. 2003). Indeed it has been shown that multiple cell-surface interactions giving an affinity greater than 10 micromolar cannot be disrupted without damaging membranes (Leckband, Muller et al. 1995). Thus, low-affinity interactions may be useful, for

example, in slowing down a fragile corpse in the circulation by phagocytic epithelial cells.

1.2.7 Examining and explaining apoptotic cell clearance: *Consensus and confusion*

It is clear that different phagocytes (professional/non-professional, stimulated/activated or unactivated, lineage origin) use different receptor systems to accomplish apoptotic-cell clearance (Fadok, Laszlo et al. 1993) and that the vast numbers apoptotic cells generated *in vivo* are scarcely detected. Yet, it is not clear why many of the phagocytic receptors implicated in this process recognise structures that appear only on cells during later stages of death. Paying detailed attention to the type of phagocyte, the type of apoptotic cell (Hart, Jackson et al. 2003), how death is induced (Wiegand, Corbach et al. 2001), at what stage in the death programme the feed cell is at, how long the phagocyte and feed are left co-incubated, the environment in which interaction is allowed to take place in (Mevorach, Mascarenhas et al. 1998), may provide an explanation as to why there are conflicting views on issues such as the phase of apoptosis (i.e."early" vs. "late") reached before cells are engulfed (Gregory and Devitt 2004) (Devitt, Pierce et al. 2003).

1.2.7.1 The "standard" phagocytosis/interaction assay

Whilst some authors report preferential clearance of "late apoptotic" cells *in vivo* (Devitt, Pierce et al. 2003) others observe clearance during earlier phases (Jersmann, Dransfield et al. 2003). The way in which an apoptotic cell interaction assay is performed and recorded may have dramatic effects on the apparent outcome of a given experiment. Let us examine some of the features of these assays, using a

monolayer of HMDMs in a 24-well format as an example (Fadok, Voelker et al. 1992). Wells of this size typically hold a maximum of 70,000 mature macrophages and commonly, between 1×10^6 and 1×10^7 feed cells are added potentially providing between 15 and 150 feed cells per macrophage. What is more, commonly, not all HMDMs within a given population appear "competent to eat" which further increases the ratio of feed cells to phagocytically competent macrophages. Now, considering the stage of death amongst the feed cell population - cultures of early apoptotic cells are often described as being >95 % negative for the uptake of vital dyes before an assay. However 5 % in a population of only 2×10^6 provides every macrophage with atleast one membrane compromised cell and given that cells are likely to progress to later stages of death through their handling and during the period of the assay the number of "late apoptotic" or "secondarily necrotic" cells available to each macrophage may be even higher by the end of the interaction. Such an effect will be more marked when assays are extended for long incubation periods, which may explain the results of Kurosaka and colleagues who observed inefficient engulfment by macrophages following a 1 hour co-culture with 6 hour post apoptotic cells compared to 3 hour co-culture with cells 4 hours post induction (Kurosaka, Takahashi et al. 2003). Additional factors, such as the shear force imparted during wash steps and the temperature at which they are carried out may explain differences in the resulting number of bound or phagocytosed cells recorded following an assay. "Extensive washing" (Devitt, Pierce et al. 2003) (6 assertive plunges into PBS) may remove more bound feed cells (and macrophages) than "gentle washing" (Eda, Yamanaka et al. 2004) of loosely bound cells. Furthermore, the inclusion or exclusion of serum may have profound effects through opsonisation of the apoptotic

cell or activation state of the macrophage (Takizawa, Tsuji et al. 1996). However, although apparently conflicting results in the literature may be a consequence of varying assay conditions, such assays may well be measuring interactions pertinent to those found *in vivo,* but simply in different contexts.

1.2.7.2 Defining the type of clearance being modelled

In vivo, the conditions surrounding an apoptosing muscle cell found deep within its closed tissue (lacking abundance of serum) are likely to be very different to those of a senescing neutrophil in the vasculature, with a consequent effect on the mechanisms used for phagocytosis. Such differences could be further exaggerated *in vitro* where following induction, apoptosis is usually left to ensue with cell cultures incubating statically. It may be that the "enzymatic" changes occurring inside a dying cell *in vitro* cell do not automatically result in the membrane changes found *in vivo*, where a dying cell is exposed to additional factors such as compressive forces through normal bodily movement or constant shear forces in the blood. Refinement of apoptotic-cell recognition assays to measure specific aspects of clearance (such as that developed by Brown *et al* see also section 1.2.5.2.1.5.1) (Brown, Heinisch et al. 2002) may lead to a greater understanding of the kinetics of clearance.

1.2 Phage antibody display technology

The following sections aim to provide background information to one of the technologies, namely phage antibody display, employed for the work described in this thesis aimed at identifying novel markers of apoptosis.

1.2.1 Bacteriophage-displayed recombinant antibody fragments

Due to the almost limitless permutations of amino acid arrangements that are possible within an antigen-binding site, antibodies can, in theory, be produced to interact with almost any substance. In addition to their role engineered by evolution, as part of the immune system in facilitating the clearance of their specific antigen, antibodies have become invaluable for medical and laboratory applications.

Recombinant DNA technology has allowed the engineering of antibodies which overcome both the inherent disadvantages of using polyclonal antisera such as unexpected cross-reactions, and the formation of "human anti-mouse antibody" (HAMA) complexes following therapeutically or diagnostically administered mouse monoclonal antibodies. With "recombinant antibody technology" antibodies are no longer produced in mammals by immunisation with antigen, but in bacteria or cell cultures, *in vitro*. To obtain higher yields only the antigen-binding portion of the antibody is produced.

Prior to recombinant technology, the smallest possible antigen-binding unit obtainable was the Fab fragment, created by protease digestion of normal antibodies. However the smallest molecule that retains the antigen-binding site, named variable region fragment (Fv), consists of only the variable regions of the light and heavy chains (figure 1-5). These must be stabilised by a peptide link, joining the variable

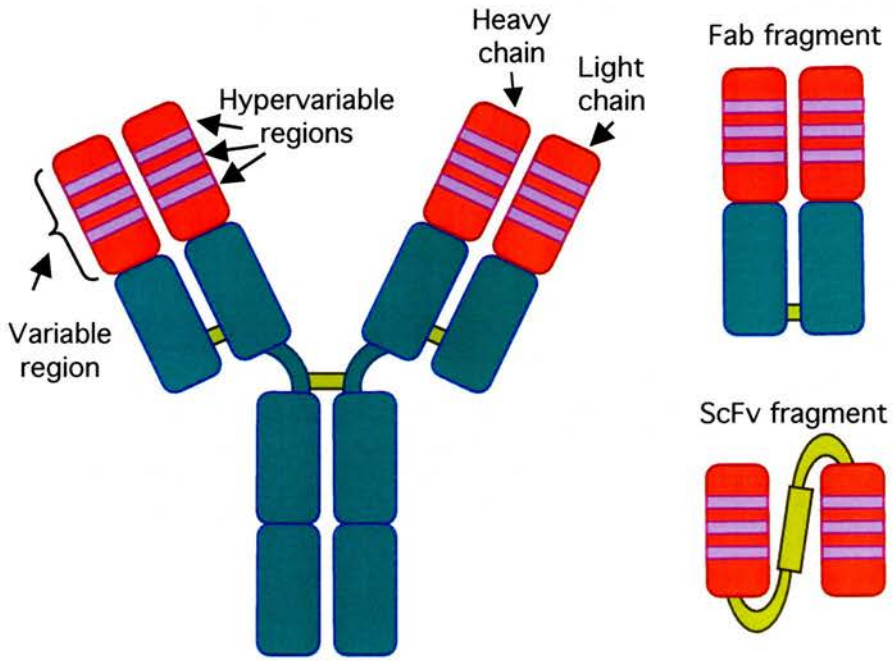


Figure 1-5. Schematic representation of an immunoglobulin antibody and antigen-binding fragments. ScFv; single-chain variable region.

domains forming a single-chain Fv fragment (ScFv).

The expression of these antibody fragments on the surface of bacteriophage (virus particles that infect bacteria) as fusions to their coat proteins provides a physical link between the antibody product and the gene that codes for it. By displaying libraries of these Ab fragments in this way it is possible to mimic the humoral immune system in bacteria. Most recombinant antibody technology using phage display uses the non-lytic filamentous phage M13 or the closely related phage fd. The antibody fragments can be fused to the N terminus of the minor coat protein 3 (gp3) of the phage or to the C-terminal domain of gp3 (Barbas, Kang et al. 1991) as Fab fragments or as ScFv fragments.

1.3.2 Sources of antibody library complexity

Combinatorial libraries of antibody fragments rely on the ability to clone antibody heavy and light fragments independently and recombine them in the desired expression system. Repertoires of antibody fragments were first generated by polymerase chain reaction (PCR) from rearranged V-genes (Orlandi, Gussow et al. 1989) of populations of lymphocytes, from the spleens of immunised mice, blood of immunized humans, and the bone marrow of infected humans (Barbas, Kang et al. 1991) leading to isolation of antibodies with excellent binding affinities (Hoogenboom, Griffiths et al. 1991). Repertoires can also be built from human V-gene segments rearranged *in vitro* ("synthetic repertoires") yet these often yield antibodies of only moderate binding affinity compared with immune repertoires of the same size. However higher affinity antibodies can be obtained by random mutation or chain shuffling (Riechmann and Winter 2000) of antibodies selected from these repertoires.

1.3.3 Selecting the desired phages

The process of selection to extract members of a phage library with desired binding properties is crucial with affinity-based selections (often referred to as biopanning) being the most common (Marks, Hoogenboom et al. 1991; Hoogenboom, de Bruine et al. 1998). Typically the selective agent (antigen) is incubated with the phage library to allow binding between the target and the appropriate phage. After a suitable incubation time, unbound phages are removed and washing steps are done to remove non-specifically bound phage. The elution is necessarily harsh since the objective is to recover members of the library with the highest affinity. As required, eluted phage can be amplified, and repeated selection cycles undertaken until enrichment of the desired phage is observed.

Despite there being no published reports of phage-displayed libraries being used to select for apoptosis-specific markers, antibodies with specificity towards viable cell surface molecules, such as epidermal growth factor (EGF) receptors, CD14, CD98 and CD40, have successfully been obtained using this approach (Schier, Bye et al. 1996; Pierce 2000; Itoh, Inoue et al. 2001; Ellmark, Ottosson et al. 2002).

1.4 Purpose of this study

The work carried out in this thesis centres around the aim of improving our understanding of how innate mechanisms recognise dying self. Using two antibody-based strategies, attempts are made to probe the apoptotic-cell surface for previously uncharacterised engulfment ligands. Initially a phage-displayed antibody library containing a repertoire of $\sim 10^8$ antibody fragments encoded by human germline genes was utilised as an unbiased source of binding specificity. An alternative approach was based on the knowledge that receptors used by macrophages to recognise apoptotic cells also recognise microbial epitopes. Looking for the ability of antibodies raised against microbe-associated molecules to cross-react with apoptotic cells in this way could help confirm or refute the hypothesis that ACAMPs revealed by cells undergoing apoptosis present molecular patterns that resemble PAMPs.

2 Materials and Methods

Chemicals and Reagents

4,6-diamidino-2-phenylindole(DAPI)	Sigma
4-well glass slides	Hendley, Essex
Acetic acid	BDH Chemicals
Acrylamide	Biorad
Agarose	Sigma
Alexafluor-568-conjugated Phalloidin	Invitrogen
Ammonium chloride	Sigma
Ammonium persulphate (APS)	Sigma
Ampicillin	Sigma
Annexin V-(FITC conjugated or biotinylated)	BioWhittaker
BactoTM agar	BDH Chemicals
Biorad Protein assay	Biorad
Biotinylation Kit	Sigma
Boric acid	Sigma
Bovine serum albumin(BSA)	Sigma
Bromophenol blue	Sigma
Butanol	BDH Chemicals
Caesium chloride	Sigma
Complement C1q	Sigma
Cycloheximide	Sigma
DePeX mounting medium	BDH Chemicals
Dextran 500	Amersham Biosciences
Dihydroxyvitamin D3	Leo-pharma, Belgium
Dimethyl sulphoxide(DMSO)	Sigma
ECL reagents	Amersham Biosciences
ECL reagents	Amersham Biosciences
EDTA	Fischer
EndoTrap-Endotoxin removal system	Profos, Germany
Ethanol	BDH Chemicals
Ethidium bromide	Sigma
Ethylenediaminetetra-acetic acid disodium salt(EDTA)	Fischer
Etoposide	Sigma
Formaldehyde	BDH Chemicals
Gene pulser electroporation cuvettes (0.4cm)	BioRad
Giemsa stain	BDH Chemicals
Glycerol	Sigma
Glycine	Fischer
Hydrochloric acid	Fischer
IntraStain	Dako
Ionomycin	Calbiochem
Jenner stain	BDH Chemicals
Kanamycin	Sigma
Laminin from human placenta	Sigma
Lipopolysaccharides	Sigma
Limulus Amebocyte Lysate endotoxin assay	BioWhittaker
Mowiol	Calbiochem
N,N,N',N'-tetramethylethylenediamine (TEMED)	Sigma
N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfionic acid](HEPES)	Sigma

Non-fat skimmed milk powder	Tesco
Percoll	Amersham Biosciences
Phorbol 12-myristate 13-acetate(PMA)	Sigma
Polyoxyethylene sorbitan monolaurate (Tween 20)	Sigma
Polyoxyethyleneglycol (PEG-8000)	Sigma
Ponceau S solution	Sigma
Potassium acetate	Sigma
Potassium chloride	Fischer
Propidium iodide	Bender Medsystems
Protease inhibitor cocktail	Sigma
PVDF membrane	Amersham Biosciences
QuikChange'R' site-directed mutagenesis kit	Stratagene
SDS	Sigma
Sodium acetate	Sigma
Sodium chloride	Fischer
Sodium citrate	Sigma
Sodium hydroxide	Fischer
Sodium phosphate dibasic anhydrous	Fischer
Sodium phosphate monobasic anydrous	Fischer
Tetracyclin	Sigma
TRAIL	Affiniti research products
Tri-reagent	Sigma
Tris(hydroxymethyl)methylamine(Tris)	BDH Chemicals
Trisma base	Fischer
Triton-X-100	Biorad
Tryptone peptone	BDH Chemicals
Yeast extract	BDH Chemicals
ZBoc-Fmk	Calbiochem

Cell culture

Dulbecco's modified eagle medium(DMEM)	Gibco
Foetal Bovine serum(FBS)	Biowhittaker
G418 sulphate	Gibco
Goat-anti-mouse coated beads	Sigma
Hybridoma medium animal component free	Sigma
Iscoves modified Dulbecco's medium (IMDM)	Gibco
L-Glutamine	Gibco
Nickel-NTA paramagnetic agarose beads	Qiagen
Non essential amino acids	Gibco
Normal goat serum(NGS)	Harlan Sera-labs
Penicillin-streptomycin(P/S)	Gibco
RPMI 1640 medium	Gibco
Serum supreme	Biowhittaker
Trypsin-EDTA (T/E)	Gibco
X-vivo-10	Cambrex

Molecular biology

10 mM dNTP solution	Invitrogen
Big-Dye Terminator sequencing Kit	Applied Biosystems
DNA 100bp ladder	Invitrogen
DNA 1Kb ladder	Invitrogen
DNA oligonucleotide Primers	MWG-Biotech
Dnase	Invitrogen
Bac-to-Bac aculovirus Expression Sytsem	Invitrogen
HisTrap nickel-NTA kit	Amersham Biosciences
HiTrap Protein G column kit	Amersham Biosciences
pcDNA3.1 Directional TOPO expression Kit	Invitrogen
Pfu polymerase	Promega
pHAT11	clontech
pSecTag2/HygroA	Invitrogen
pTrcHis2C	clontech
Qiaquick gel extraction kit	Qiagen
Restriction enzymes	Promega
Reverse Transcriptase	Promega
RNase A	Promega
Rnasin	Promega
T4 ligase	Invitrogen
Taq polymerase	Roche, USA

Antibodies

Name	antigen	species	class/ isotype	obtained from
Cell-surface				
Bu65	**ICAM-3**	mouse mono	IgG	Birmingham University
61D3	**CD14**	mouse mono	IgG 1	Birmingham University
63D3	**CD14**	mouse mono	IgG 1	Birmingham University
MLuc5	**67 kDa LBP/p40**	mouse mono	IgM	Stratech
Intracellular				
AC15	**B-actin**	mouse mono	IgG 1	sigma A5441
B-tubulin(DM-1B)	**B-tubulin**	mouse mono	IgG 1	neomarkers
KS20.8	**cytokeratin-20**	mouse mono	IgG 2a	DAKO M7901
LamR (F-18)	**LBP/p40**	goat polyclonal	-	Santa Cruz
Non-eukaryotic				
wn1 222-5	**LPS**	mouse mono	IgG 2a	Edinburgh University
R1	**R1 LPS**	mouse mono	na	Edinburgh University
R2	**R2 LPS**	mouse mono	na	Edinburgh University
R3	**R3 LPS**	mouse mono	na	Edinburgh University
F6 446-24	**LPS**	mouse mono	na	Edinburgh University
F6 504-11	**LPS**	mouse mono	na	Edinburgh University
F6 514-12	**LPS**	mouse mono	na	Edinburgh University
FK4 224.10 10	**LPS**	mouse mono	na	Edinburgh University
FK4 224-14	**LPS**	mouse mono	na	Edinburgh University
FK4 224-17	**LPS**	mouse mono	na	Edinburgh University
FK4 224-24	**LPS**	mouse mono	na	Edinburgh University
UT	**LPS**	mouse mono	na	Edinburgh University
MOMP	**Chlamydial MOMP**			
MOMP	**Chlamydial MOMP**	goat polyclonal	-	Edinburgh University
15306	**LPS**	mouse mono	IgG 2a	QED biosciences
15308	**LPS**	mouse mono	IgG 3	QED biosciences
15174	**LPS**	mouse mono	IgG 2a	QED biosciences
Ad x	**coat protein Ad5**	mouse mono	na	Edinburgh University
Ad 45	**coat protein Ad5**	mouse mono	na	Edinburgh University
a-M13	**M13 gp8**	mouse mono	IgG 2a	Pharmacia
Control antibodies				
mouse IgG 2a	-	-	IgG 2a	Sigma
mouse IgG1	-	-	IgG 1	Sigma
mouse IgG 3 lambda	-	-	IgG 3	Sigma
Detection antibodies				
anti-His	**6xHistidine**	mouse mono	IgG 2b	Invitrogen
a-V5	**V5 epitope**	mouse mono	IgG 2a	Invitrogen
goat-anti-mouse IgG FITC	**mouse Ig**	goat polyclonal	-	Sigma
goat-anti-mouse HRP	**mouse Ig**	goat polyclonal	-	Amersham Biosciences
goat-anti-mouse alexa fluor conjugates	**mouse Ig**	goat polyclonal	-	Invitrogen

Equipment

Equipment	Supplier
Centrifuges	
Beckman GS-6R	Beckman, UK
Beckman J2-HS with JA14 and JA21rotors	Beckman, UK
Rotina 46-R	Hettich, Germany
Sigma 1-15K	Sigma, USA
Cell culture	
BS748 improved neubauer haemocytometer	Weber, UK
Incubator	LEC,UK
Microflow class II hood	Walker safety cabinets, UK
Orbital shaking incubator	SANYO Gallenkamp, UK
Static incubator	SANYO Gallenkamp, UK
Microscopes	
Flourescence microscope , Axioskop 2	Zeiss , Switzerland
Leica inverted TCS-NT microscope	Leica Microsystems, UK
Leica upright TCS-NT microscope	Leica Microsystems, UK
Olympus CK2 light microscope	Olympus, Japan
TCS-NT laser scanning unit	Leica Microsystems, UK
General	
800 W microwave	Proline, UK
Anthos lt-2 plate reader	Anthos Labtec Instruments , Austria
Cecil-200 spectrophotometer	Cecil instruments, UK
Coulter EPICS XL-MCL flow cytometer	Beckman Coulter, USA
Conical polypropylene tubes (15ml and 50ml)	BD Biosciences, UK
Tissue cell culture flasks and plates	Nunc International, UK
Decon sonicator	Decon labs, UK
Gene Pulser TM Electroporation apparatus	Biorad, UK
Grant waterbath	Grant instruments, UK
Horizon gel tanks (size 58 and 11.4)	Life Technologies, USA
Mini protean II gel system	Biorad, USA
Peristaltic pump , type 505S	Watson Marlow , Falmouth , UK
Power Pac 300	Biorad, UK
PTC-100 thermal cycler	GRI, UK
Transilluminator	UV products Ltd, UK
Computer packages	
Amplify 1.0	Bill Engels, University of Wisconsin
Flo-Jo v 4.4.1	Tree-Star, USA
OpenLab	Improvision, UK

2.1 Molecular Cloning

All DNA manipulations were carried out as described in (Sambrook, Fritsch et al. 1989).

2.1.1 Plasmid vectors used in this work

Refer to Fig. 2-1 for maps of plasmid vectors used for DNA cloning during this work.

2.1.2 Extraction and purification of plasmid DNA from bacterial cells

Plasmid DNA was purified using alkaline lysis followed by alcohol precipitation as described below.

2.1.2.1 Small scale preparations of plasmid DNA

1.3ml of a 5ml overnight bacterial culture was placed in a sterile Eppendorf tube and pelleted at 13000g for 2 minutes to pellet the cells. After discarding the supernatant cells were resuspended in 200μl of glucose/Tris/EDTA buffer (GTE, see appendix 2-1), to which an additional 400μl of 1%SDS/0.2M NaOH was added. The solution was mixed by inversion and then left for 5 minutes during which time cell lysis occurred. Following lysis 300μl of potassium acetate buffer ($3MK^+/5MAc^-$, see appendix 2-1) was added per tube before mixing by inversion. The resultant white precipitate was pelleted by centrifugation at 13,000g for 7 minutes before adding the supernatant to a fresh sterile Eppendorf containing 600μl of isopropanol. Tubes were mixed by inversion before centrifugation at 14,000g for 5 minutes to pellet the

DNA. The supernatant was discarded and the pellet washed in 500µl 80% ethanol, before allowing to air dry. The final pellet was resuspended in 30µl of dH_2O or Tris/EDTA buffer (TE, see appendix 2-1).

2.1.2.2 Large scale preparations of plasmid DNA

500µl of a 5ml bacterial culture (grown overnight) was added to 100ml of LB containing the appropriate antibiotics and allowed to grow overnight at 37°C. The following day cells were pelleted from the culture by centrifugation at 6,000g for 10 minutes in sterile 300ml centrifuge tubes (Nalgene). The pellet was resuspended in 12ml of GTE to which an additional 16ml of 1%SDS/0.2M NaOH was added. The two were mixed by inversion and left for 5 minutes to allow lysis to occur. Following lysis 18ml of $3MK^+/5MAc^-$ was added and the solution mixed by inversion. After 5 minutes incubation on ice the resultant white precipitate was pelleted at 10,000g for 10 minutes and the supernatant added to 40ml of ice-cold isopropanol. After a further 5 minutes on ice the DNA preciptate was pelleted in a fresh sterile centrifuge tube at 10,000g for 15 minutes. The resultant pellet was resuspended in 500µl of TE before adding RN-ase A (Promega) to 80µg/ml and incubating at 37°C for 45 minutes. Finally the sample was phenol/chloroform extracted before storage at –20°C.

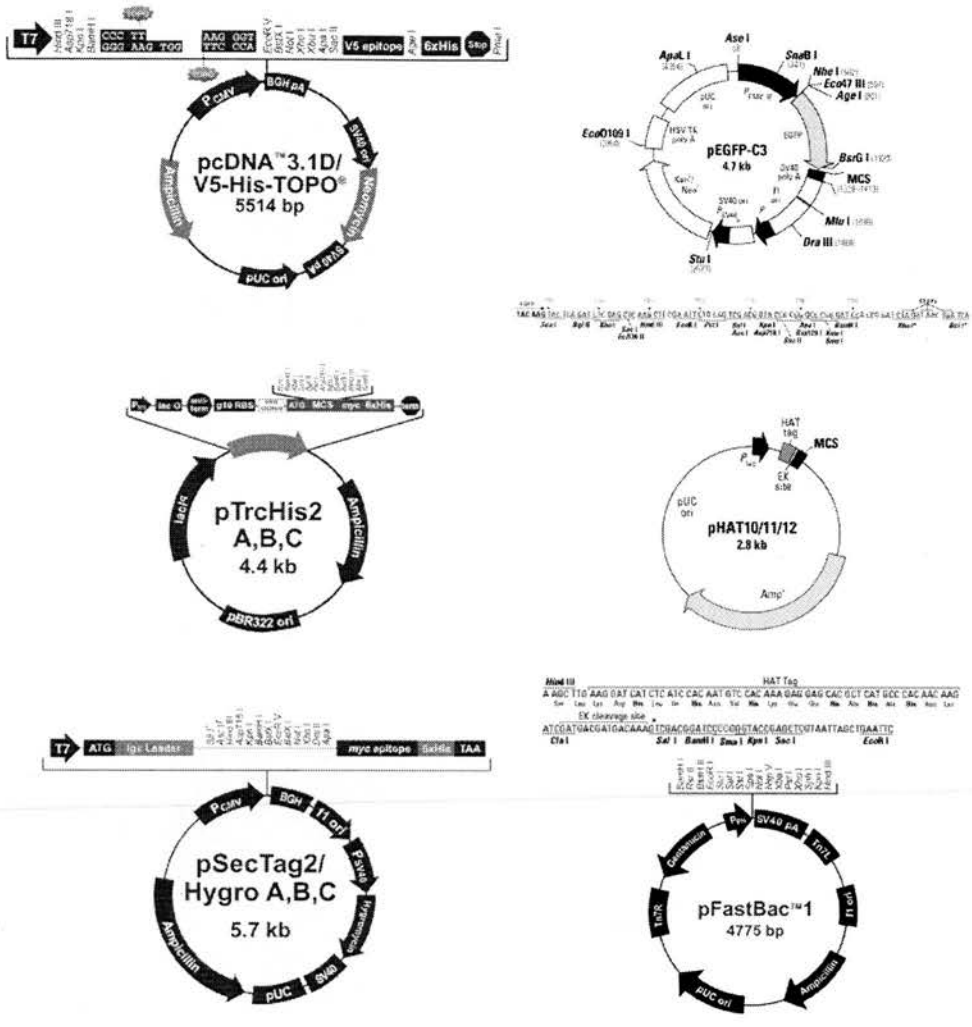


Figure 2-1. Maps of plasmid vectors used for DNA cloning during this work.

2.1.3 Strategies for cloning in plasmid vectors

2.1.3.1 Ligation of plasmid DNA

Following digestion with the appropriate restriction endonucleases, DNA samples were separated by electrophoresis in a 1% agarose gel. The amount of DNA in a sample was quantified by visual comparison of band intensity with a standard. The required DNA fragments were excised and DNA extracted from the gels by silica-based purification using the QIAquick® Gel Extraction Kit (QIAGEN) according to manufacturer's instructions. Considering that the in-gel method used to quantify the DNA was not entirely precise, three ligation reactions with varying ratios of vector DNA to foreign insert DNA were assembled in separate sterile Eppendorf tubes, as detailed in table 2-1. The ligation reactions were allowed to proceed for between 4-16 hours at 16-21°C.

2.1.3.2 Preparation and transformation of competent *Escherichia coli*.

Refer to table 2-2 for the specific strains of *Escherichia coli* (*E.coli*) used for individual cloning applications.

Below are typical protocols for preparation and transformation of competent *Escherichia coli*.

2.1.3.2.1 Preparation of competent E. coli.

A single colony of *E. coli* was allowed to grow to saturation in 5mls of LB overnight at 37°C. 1ml of the resultant overnight culture was added to 50mls of fresh LB. The culture was grown until a 0.4-0.5 OD_{600} was reached, then incubated on ice for 10

minutes. Cells were then harvested at 2000g for 20 minutes before resuspending in 25ml of ice cold 0.1M $CaCl_2$ and incubating on ice for 30 minutes. Subsequently the cells were centrifuged at 2000g for 20 minutes before resuspending in 5ml of ice-cold 0.1M $CaCl_2$/15% glycerol and a further 30 minute incubation on ice prior to use.

2.1.3.2.2 Transformation of competent **E. coli** *with plasmid DNA*

5-10μl of the ligation reaction was added to 100μl of freshly prepared competent cells. Tubes were left on ice for 30 minutes and then heat shocked at 42°C for 30-120sec depending on bacterial strain. 250μl of SOC medium (NZY for Xl1 Blue) was added to each tube and incubated with shaking at 37°C for an hour to allow expression of the antibiotic resistance gene. 200μl of the bacterial culture was spread onto LB agar plates containing the appropriate selective antibiotic(s) before overnight incubation at 37°C.

On each occasion the following two control transformations were included; (a) Competent cells without plasmid DNA and (b) Competent cells plus a known amount of closed circular plasmid DNA lacking the desired insert.

	Molar ratio - vector DNA:foreign DNA		
	1:3	1:1	3:1
Vector DNA (ng)	25	50	75
Foreign insert DNA (ng)	75	50	25
T4 DNA ligase (Weiss units)	1	1	1
Nuclease free water	to 20 µl	to 20 µl	to 20 µl

Table 2-1. Reaction components for ligation of plasmid DNA.

E. coli strain	Application	Heat shock time (seconds)	Methods section refernce
TOP10	cDNA cloning into pCDNA3.1D/V5-His-TOPO and subcloning into pSecTag2/HygroA and pFastBac1	30	2.1.8 2.1.10.3 2.1.10.4
XL1-Blue	Site directed mutagenesis of pCDNA3.1D/LBP/p40-V5-His and phage display	120	2.1.9 2.12
MC1061/p3	Subcloning into pHAT11 and pTrcHis2C	30	2.1.10.2
SCS110	Subcloning into pEGFP-C3	120	2.1.10.1
DH10Bac	Generation of recombinant Baculorius	45	2.1.10.4
TG1	Phage display	na	2.12

Table 2-2. E.coli strains used for transformation of vectors. Refer to materials and methods section 2.1.2.2.1 for a general protocol for preparation of competent E. coli.

	Volume
25mM $MgCl_2$	4µl
RT transcription buffer	2µl
10mM dNTP mixture	2µl
RNAse inhibitor	0.5µl
oligo dTs	0.5µl
AMV RT enzyme	2µl

Table 2-3. Components of the reaction used for reverse transcription (RT)

2.1.3.3 Identification of bacterial colonies that contain recombinant plasmids by restriction analysis

A small number of colonies (typically 5-20) from those returning from a transformation were used to inoculate 5ml of LB containing the appropriate antibiotic and grown overnight at 37°C. The resultant cultures were used for small-scale isolation of plasmid DNA, and analysed by digestion with restriction enzymes and electrophoresis through a 1% agarose gel containing 0.5μg/ml ethidium bromide and visualised under UV light (254-366 nm).

2.1.3.4 Strategies for cDNA cloning

2.1.3.4.1 Preparation of mRNA

$1x10^7$ cells were harvested by centrifugation and washed twice in ice cold PBS. The final pellet was resuspended in 1ml of TRI-Reagent™, left to stand at RT for 5 minutes before adding 200μl chloroform and vortexing gently for 15 seconds. Following a further 10 minutes incubation at RT samples were centrifuged at 13,000g for 15 minutes at 4°C. The aqueous phase was removed and added to 500μl of isopropanol. The solution was mixed by inversion and allowed to incubate for 10 minutes at RT before pelleting the precipitated RNA by centrifugation at 13,000g for 10 minutes at 4°C. The resultant pellet was washed with 1ml of 75% ethanol before air-drying. Pellets were dissolved in 50μl of nuclease free dH_2O before use or stored at -80°C. The quality and quantity of RNA was assessed by electrophoresis through a 1% agarose gel containing 0.5μg/ml ethidium bromide and visualised under UV light (254-366 nm).

2.1.3.4.2 Reverse transcriptase synthesis of cDNA

To 5μg of mRNA was added 0.5μl RNAse inhibitor and nuclease free dH_2O to 7μl. Samples were heated for 5 minutes at 95°C to denature the RNA then placed on ice whilst the reaction components (detailed in table **x**) were added. The reaction was incubated at 42°C for 1 hour. In order to check for contamination by genomic DNA a control reaction containing the same components with the exception of AMV reverse transcriptase (RT) was included for each mRNA sample. Following transcription the enzyme was heat inactivated at 95°C for 5 minutes before adding 80μl of nuclease free dH_2O. Sample quality was assessed by PCR amplification of the DNA encoding GAPDH. Table 2-4 lists primer sequences and table 2-5 provides details of a typical programme for PCR (see also section 2.1.4).

2.1.4 Amplification of DNA by the polymerase chain reaction (PCR)

Refer to table 2-4 for details of specific primer sets for PCR. To aid design of primers that minimised the chance of primer dimer formation and that possessed suitable melting temperatures the PCR outcome was simulated using the *Amplify 1.0* software (University of Wisconsin). When PCR products were to be used for cloning, amplification reactions were carried out using *Pfu* Polymerase (QIAGEN) or for Site directed Mutagenesis *Pfu Turbo* (Stratagene)

Table 2-5 details a typical programme used for amplification of DNA in a PTC-100 thermal cycler (MJ Research INC).

primer name		expected size (bp)	sequence 5'-3'	annealing temp(oC)
pCDNA3.1D/LBP/p40-V5-His	for rev	891	CACCATGTCCGGAGCCCTTGAT AGACCAGTCAGTGGTTGCTCCTAC	59
pCDNA3.1D/NEDD5-V5-His	for rev	1049	CACCATGTCTAAGCAACAGCCAACTCAG CACGTGGTGCCCGAGAGCCCCG	58
pCDNA3.1D/LBP/p40-pm	sense antisense	complete vector	CCTGGGAGAAGCTCCTGCTGGCAGCTCG CGAGCTGCCAGCAGGAGCTTCTCCCAGG	55
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	for rev	528	CGACAGTCAGCCGCATCTTCTTTTGCGTC GGACTGTGGTCATGAGTCCTTCCACGATA	59
pCDNA3.1D/LBP/p40-V5-His M13	for rev	~1600	CACCATGTCCGGAGCCCTTGAT GGAAACAGCTATGACCATG	55
Fdseq M13	for rev	~1000	GAATTTTCTGTATGAGG GGAAACAGCTATGACCATG	50
T7 BGH	for rev	Sequencing Sequencing	TAATACGACTCACTATAGGG TAGAAGGCACAGTCGAGG	50

Table 2-4 Primer sets for PCR amplification of DNA. Listed are the annealing temperatures used and expected products sizes in base pairs (for; forward primer, rev; reverse primer, bp; base pairs).

2.1.5 Cloning of LBP/p40 and NEDD5 cDNA into the pCDNA3.1D/V5-His-TOPO© vector

The pCDNA3.1© Directional TOPO ® Expression Kit (Invitrogen) was used for directionally cloning blunt-end PCR products into pCDNA3.1D/V5-His-TOPO© vector.

Table 2-4 lists the combined primer sets, pCDNA3.1D/LBP/p40-V5-His and pCDNA3.1D/NEDD5-V5-His used for amplification of laminin-binding protein (LBP/p40) and NEDD5 genes respectively, from Mutu I cDNA. The oligonucleotide primer sequences were designed to amplify the complete coding sequence of the genes cited in the records obtained from the primary result of MALDI-TOF analysis for LBP/p40 (Yow, Wong et al. 1988) and NEDD5 (Strausberg, Feingold et al. 2002) (accession no.s X61156.1 and BC014455.1 respectively). The addition of four bases, CACC, to the 5' end of forward primers allowed PCR products to be directionally cloned into the pCDNA3.1D/V5-His-TOPO© vector. In order to fuse the C-terminus of the open reading frames for each gene in-frame with the V5 epitope and 6xHis tag contained within the pCDNA3.1 vector, reverse primers were designed to exclude the stop codons of their corresponding genes.

The resulting plasmids were named pCDNA3.1D/LBP/p40-V5-His and pCDNA3.1D/NEDD5-V5-His, the multiple cloning sites (MCSs) of which are represented in figure 2-2.

Following identification of plasmid DNA containing inserts of the expected size by restriction analysis (section 2.1.3.3), sequences were confirmed as described in section 2.1.8 employing primer sets specific for sites flanking the LBP/p40 cDNA insert (T7 and BGH, Table 2-4).

2.1.6 Site directed mutagenesis of cloned LBP/p40 DNA

A QuikChange® mutagenesis kit (Stratagene) was applied for removing the internal Hind III consensus sequence by introducing a silent point mutation at the position corresponding to nucleotide 140 of the LBP/p40 gene (accession no.s X61156.1.) according to manufacturers' instructions. The PCR programme, and components of the reaction used for site directed mutagenesis are detailed in table 2-6 and 2-7 respectively.

The plasmid pCDNA3.1D/LBP/p40-V5-His was used as a template for constructing the point mutation through PCR. The primer pair pCDNA3.1D/LBP/p40-pm (Table 2-4) was synthesised to exchange change codon CTT (bps 142-144 -leucine) for codon CTC (leucine). The resulting plasmid was named pCDNA3.1D/LBP/p40-pm. Mutations were confirmed by sequencing as described in section 2.1.8 employing primer sets specific for sites flanking the LBP/p40 cDNA insert (T7 and BGH, Table 2-4).

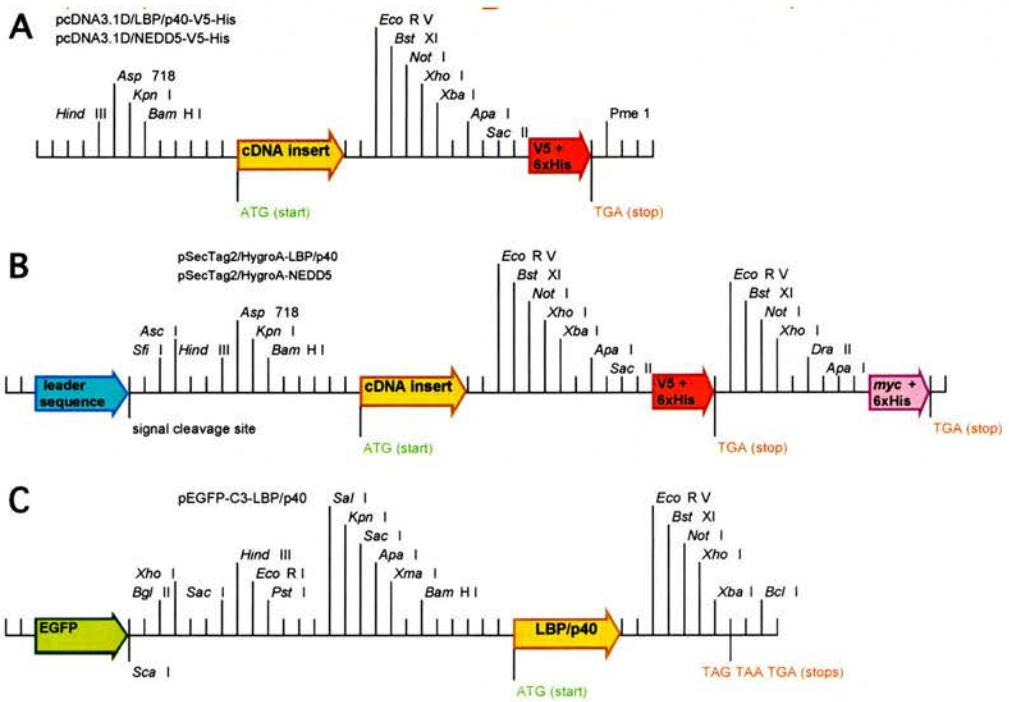


Figure 2-2. Schematic representations of multiple cloning sites for recombinant plasmids produced during this work (mammalian expression systems). See figure 2-1 for maps of parental plasmid vectors.

A; pCDNA3.1D/LBP/p40-V5-His and pCDNA3.1D/NEDD5-V5-His, see section 2.1.4

B; pSecTag2/HygroA-LBP/p40 and pSecTag2/HygroA-NEDD5 see section 2.1.6.3.

C; pEGFP-C3-LBP/p40, see section 2.1.6.1.

2.1.7 Subcloning of LBP/p40 and NEDD5 DNA

2.1.7.1 Production of LBP/p40 as a green fluorescent protein (GFP) fusion construct

DNA containing the LBP/p40 insert was isolated from pCDNA3.1D/LBP/p40-V5-His by double digestion with *Bam*HI and *Xba*I and further subcloned into the pEGFP-C3 (Clontech) at the same restriction sites. The MCS of the resulting plasmid, which was named pEGFP-C3-LBP/p40, is represented schematically in figure 2-2.

2.1.7.2 Subcloning of LBP/p40 for expression in a bacterial expression system.

The DNA insert containing the LBP/p40 plus V5 and 6xHis-tag coding sequences was isolated from pCDNA3.1D/LBP/p40-pm by double digestion with *Hin*dIII and *Pme*I and further subcloned into the pHAT11 (Clontech) digested with *Hin*dIII and *Sma*I.

The MCS of the resulting plasmid, which was named pHAT11-LBP/p40-pm, is represented schematically figure 2-3.

This vector introduced an *Eco*RI restriction site allowing further subcloning of the insert LBP/p40 plus V5 and 6xHis-tag coding sequences into the vector pTrcHis2C (Invitrogen) for high level expression in a bacterial system. This was achieved by double digestion of pTrcHis2C and vector pHAT11-LBP/p40-pm with *Bam*HI and *Eco*RI. The MCS of the resulting plasmid, which was named pTrcHis2C-LBP/p40-pm, is represented schematically in figure 2-3.

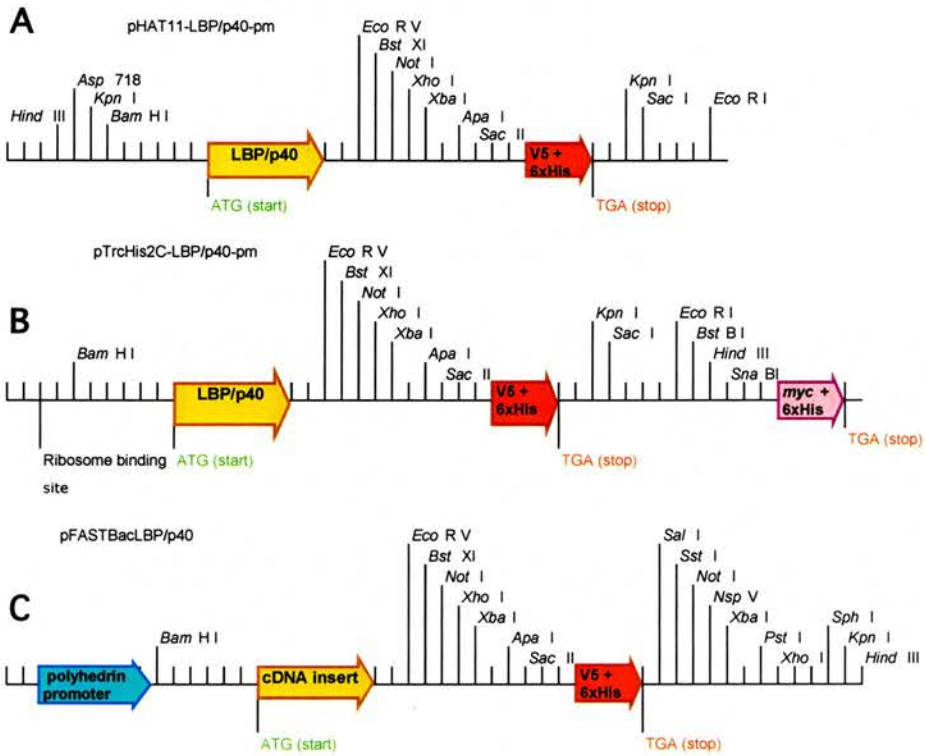


Figure 2-3. Schematic representations of multiple cloning sites for recombinant plasmids produced during this work (bacterial and insect expression systems). See figure 2-1 for maps of parental plasmid vectors.
A; pHAT11-LBP/p40-pm, see section 2.1.7.2.
B; pTrcHis2C-LBP/p40-pm, see section 2.1.7.2.
C; pFastBac-LBP/p40, see section 2.1.7.4.

2.1.7.3 Production of LBP/p40 and NEDD5 constructs for expression as secreted proteins.

The DNA inserts containing the LBP/p40 and NEDD5 each with V5 and 6xHis-tag coding sequences were isolated from pCDNA3.1D/LBP/p40-pm and pCDNA3.1D/NEDD5-V5-His respectively by double digestion with *Hin*dIII and *PmeI* and then subcloned into the pSecTag2/HygroA (Invitrogen) digested with *Hin*dIII and *Eco*RV.

The MCSs of the resulting plasmids, which were named pSecTag2/HygroA-LBP/p40 and pSecTag2/HygroA-NEDD5 respectively, are represented schematically in figure 2-2.

Confirmation that target DNA had been subcloned in-frame with the leader sequence provided by pSecTag2/HygroA vector was achieved by sequencing as described in section 2.1.8 employing primer sets specific for sites flanking the DNA inserts (T7 and BGH, Table 2-4).

2.1.7.4 Production of LBP/p40 in insect cells.

The Bac-to-Bac® Expression System (Invitrogen) was used to generate recombinant baculovirus, containing the DNA encoding LBP/p40, for expression of the protein in *Spodeoptera frugiperda* Sf9 insect cells according to manufacturers' instructions.

The DNA inserts containing the LBP/p40 plus V5 and 6xHis-tag coding sequence was isolated from pCDNA3.1D/LBP/p40-V5-His by double digestion with *Bam*HI and *PmeI* and then subcloned into the pFastBac™ 1 (Invitrogen) digested with *Bam*HI and *Stu*I. The MCS of the resulting plasmid, which was named pFastBac-LBP/p40, is represented schematically in figure 2-3.

To generate recombinant bacmid DNA, DH10Bac™ *E.coli* were transformed with pFastBac-LBP/p40 (see section 2.1.3.2.2). Confirmation that target DNA had been transposed into the bacmid vector was achieved by PCR as described in section 2.1.4 employing primer sets specific for sites flanking the DNA inserts (pCDNA3.1D/LBP/p40-V5-His for and M13 rev, Table 2-4).

2.1.8 DNA sequencing

Cloned DNA was sequenced using the BigDye® v3.1 Kit (Applied Bioscience) which is based on the Sanger method (Sanger, Nicklen et al. 1977) of chain termination using fluorescently labelled dideoxynucleoside triphosphates. Reaction components were assembled according to the protocol provided by the manufacturer and the reaction performed in a PTC-100 thermal cycler (MJ Research INC). Refer to table 2-8 for details of the reaction components used for sequencing. Table 2-4 lists details of plasmid/promoter specific primers used for sequencing. 2.0µl 3M sodium acetate (pH 4.6), 50µl 100% ethanol and 10µl dH_2O was added to each of the completed reactions before incubation at room temperature for 20 minutes. The resultant DNA precipitate was pelleted by centrifugation at 13,000g for 50 minutes at 4°C. DNA pellets were washed with 70% ethanol and air dried prior to being sent for electrophoretic analysis using an ABI prism sequencer.

Step name	Step no.	Temp (° C)	time (min)
initial denaturation	1	95	2
denaturation	2	95	0.75
anneal	3	primer specific	1
extend	4	72	2
goto to step 2 (x 25)	5	-	-
cool	6	4	hold

Table 2-5 Example of a typical programme used for amplification of DNA in a PTC-100 thermal cycler (MJ Reasearch INC)

Step name	Step no.	Temp (° C)	time (min)
denaturation	1	96	0.5
anneal	2	50	0.25
extend	3	60	4
goto to step 2 (x 25)	4	-	-
cool	5	4	hold

Table 2-6 Details of the programme used for site-directed mutagenesis by PCR

Component	Amount
pCDNA3.1 V5His LBP/p40	10ng
sense primer	15pmol
antisense primer	15pmol
10x PCR Reaction Buffer	2.5μl
dNTP mix (10mM)	1μl
distilled water	To 50μl
Pfu Turbo DNA Polymerase	1μl

Table 2-7 Components of the PCR reaction used for site directed mutagenesis

Component	Amount
plasmid DNA	250ng
Primer (10pmol/ul)	0.5μl
5x sequencing buffer	2.0μl
BigDye terminator	2.5μl
distilled water	To 10μl

Table 2-8 Details of the reaction components used for sequencing using the BigDye® v3.1 Kit (Applied Bioscience)

2.1.9 Expression of cloned genes in cultured mammalian cells

2.1.9.1 Transient transfection into human embryonic kidney (HEK) 293T cells

Plasmid DNA was introduced as a coprecipitates of calcium and phosphate to resuspended HEK 293^T cells as follows:-

In a 50ml conical polypropylene tube (Becton Dickinson) air was bubbled through the 2×HBS whilst an equal volume of 0.25M $CaCl_2$ containing the desired plasmid was added drop-wise (refer to table 2-9 for the quantity of each reagents used per 1×10^6 cells. Reactions were scaled up as appropriate). The resultant mixture was incubated at room temperature for 10 minutes to allow precipitate formation before adding to the cell suspension and seeding onto tissue culture plastic or Teflon-coated 4-well slides. After 24 hours the medium was removed, the adhered cells washed with serum-free DMEM and replaced with an identical volume of fresh medium. The composition of the medium used as a replacement varied between experiments and was supplemented with varying concentrations of Serum Supreme (Cambrex) (refer to table 2-10 for details). When downstream purification of Fc-tagged recombinant proteins was intended, medium was filtered through a Hitrap™ protein G (Amersham Biosciences) column, as described in section 2.2.1 to remove bovine immunoglobulin. For production of fusion proteins, cells were left for a further 4-5 days.

2.1.10 DNA transfection by electroporation

Cells were harvested and resuspended in culture medium as listed in table 2-11. 10μg of plasmid DNA was added to an electroporation cuvette and the resuspended cells added. The cuvette was placed on ice for 10 minutes before subjecting cells to the electroporation conditions listed in table 2-11 using a Gene Pulser (Biorad). The cuvette was returned to ice for 10 minutes before transferring the cells to a $25cm^2$ tissue culture flask containing 10ml of culture medium. Electroporated cells were incubated overnight at 37°C before the addition of the appropriate drug for selection of cells stably expressing plasmid-encoded resistance genes. Prior to transfection a drug titration was carried out on each cell line to determine the optimal dose of drug to select for stable transfection. For suspension cell lines, the minimal dose that killed all cells of a non-resistant culture within 2-3 passages was chosen. For adherent cells, the minimal dose that killed all cells between 4 days and 2 weeks (dependent on cell line) was chosen.

2.1.11 Expression of cloned genes in *Escherichia coli*

1ml of a 5ml overnight culture of *E. coli* cells (TOP-10) transformed with pTrcHis2C-LBP/p40-pm was added to 250ml of LB containing ampicillin (100 μg/ml) and allowed to grow with shaking at 37°C. When OD_{600} of the culture reached 0.5, isopropyl-1-thio-ß-D-galactoside (IPTG) was added to a final concentration of 1 mM and the cells were harvested by centrifugation 4 hours later. Aliquots of total cell extracts were collected before and after induction with IPTG for analysis by SDS-PAGE and Western blotting.

Component	Volume (ml)
0.25M $CaCl_2$ containing 5mg of plasmid DNA	312.5
2xHBS	312.5
cells at $1.6x10^5$/ml in complete DMEM	6250

Table 2-9. Components of transfection reaction used to introduce plasmid DNA as a coprecipitate of calcium and phosphate to cells. Refer to methods section 2.1.8.1 for a detailed protocol.

Quantities given are those used per million cells. Reactions were scaled up as appropriate.

Protocol	Section Ref	Medium replacement
ICAM-3 Fc	4.1.3.1	1 % Protein G adsorbed SS supplemented DMEM
His-tagged ICAM-3	4.1.3.2.1	complete DMEM
His-tagged ICAM-3	4.1.3.2	Animal component free medium
His-tagged LBP/p40	7	Animal component free medium

Table 2-10 Composition of the cell culture medium used as a replacement for transfection mixtures. SS; serum supreme.

Cell type	Volts	μF	Cell density	Medium	Volume
B-JAB	650	25	$1x10^7$/ml	RPMI	600μl
MCF-7	245	960	$3x10^6$/ml	DMEM	800μl
K562	650	25	$1x10^7$/ml	RPMI	600μl

Table 2-11 Conditions used for DNA transfection by electroporation. Refer to methods section 2.1.13 for a detailed protocol

2.2 Protein Protocols

2.2.1 Purification of Fc-fusion proteins by adsorption to Protein G

Culture supernatants were harvested from HEK-293^T cells 5-6 days post transfection, and filtered through a 0.45μm pore filter before dialysing against 20mM sodium phosphate buffer (pH7.0). Supernatants were passed through a 1ml Hitrap™ protein G column (Amersham Biosciences) for purification, according to manufacturers' instructions. Firstly, the columns were equilibrated with 10ml of 20mM sodium phosphate buffer (pH7.0) prior to applying the dialysed supernatant. Secondly, the columns were washed with 10ml 20mM sodium phosphate buffer (pH7.0) to remove any unbound protein before eluting the bound protein by the addition of 0.1M Glycine-HCl (pH 2.7). 1ml fractions were collected in Eppendorfs containing 50μl of 1M Tris-HCl (pH 9.0) for neutralisation.

Fractions containing eluted protein were identified using a modified Bradford Reagent (Bradford 1976) assay (Biorad) according to manufacturers instructions. Briefly, 200μl aliquots of Biorad Reagent diluted to 20% in dH_2O and passed through a 0.45μM filter before adding to wells of an ELISA plate containing 10μl of samples. Protein containing fractions were pooled and dialysed against PBS before determining total protein concentration by comparison of their absorbance at OD_{568nm} to IgG standards in the Bradford Reagent assay. For long-term storage fusion proteins were kept at –20°C.

2.2.2 Purification of His-tagged proteins by nickel affinity.

2.2.2.1 Preparation of cell lysates

All steps were carried out at 4°C with ice-cold solutions. Cell pellets were resuspended in binding buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl,) and lysed by sonication using 5 pulses at an amplitude of 4-6 microns and at a nominal frequency of 20kHz. The insoluble debris was removed by centrifugation at 1000g for 15 minutes and the cleared supernatant passed through a 0.45μM filter prior to use for protein purification.

2.2.2.2 Preparation of culture supernatants

Culture supernatants were dialysed against binding buffer and passed through a 0.45μM filter prior to use for protein purification.

2.2.2.3 Purification

The His-Trap™ columns (Amersham Biosciences) were first equilibrated with 0.1 M nickel sulphate to charge the column with nickel ions followed by 5 column volumes of dH_2O to remove unbound nickel ions from the column, and then by 5 column volumes of binding buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl) to equilibrate the column. Imidazole was added to the prepared sample at a final concentration of 5-10μM before applying to the His-Trap column. The column was washed with 5 column volumes of binding buffer before elution of bound protein with elution buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, and 500 mM imidazole). Fractions containing protein were identified as described in section 2.2.1.

Protein-containing fractions were combined, dialysed against PBS and stored at 4°C or -20°C (long term).

2.2.3 Cell lysis and fractionation

All centrifugation and incubation steps were carried out at 4°C with ice-cold solutions.

Cells were washed twice in PBS by centrifugation at 200g for 5 minutes. The final pellet was resuspended in solubilisation buffer (10mM Hepes, pH7.4/1mM EDTA/1% TritonX-100 containing 1µl of mammalian protease inhibitor cocktail (Sigma) per million cells) and left on ice for 20 minutes before sonication using 5 pulses at an amplitude of 4-6 microns and at a nominal frequency of 20kHz. Lysates were centrifuged at 1000g for 15 minutes to pellet insoluble and nuclear material (fraction N).

The resultant supernatant was either used directly for analysis by SDS-PAGE or subjected to further fractionation by sequential centrifugation at 27,000g for 30 minutes (fraction P1) and finally100,000g for 50 minutes (fraction P2). The final supernatant (fraction S) was also reserved. Pellets were resuspended in an equal volume to that recovered from the final supernatant.

2.2.4 Production of proteoliposomes

All centrifugation and incubation steps were carried out at 4°C with ice-cold solutions.

1×10^8 cells were washed twice in PBS by centrifugation at 200g for 5 minutes. Cells were resuspended at a density of 20×10^6/ml in lysis buffer (10mM Hepes, pH7.4/1mM EDTA/1% containing 1µl of mammalian protease inhibitor cocktail

(Sigma) per million cells). Cells were lysed by 2-3 cycles of freeze-thawing before sonication using 5 pulses at an amplitude of 4-6 microns and at a nominal frequency of 20kHz. Lysates were centrifuged at 1000g for 15 minutes to pellet insoluble and nuclear material. The resultant supernatant was subjected to further fractionation by sequential centrifugation at 27,000g for 30 minutes and finally100,000g for 50 minutes. The final supernatant was removed and the pellet resuspended in a combined volume of 6ml HBSS and briefly sonicated before use or storage at -20°C.

2.2.5 SDS-polyacrylamide gel electrophoresis

Supernatants from cell lysates (prepared as described in section 2.2.2.1) were added an equal volume of 2× Laemli sample buffer and the solution heated to 95°C for 5 minutes. Samples (typically 15μl for mini-gels and ~100μl for large gels) were loaded into lanes of 10% acrylamide gels and separated by SDS-PAGE. This was achieved using the Biorad Mini-Protean II System (mini-gels) or a Sigma large vertical electrophoresis unit (large gels). Electrophoresis was allowed to proceed for 40 minutes (mini-gels) or ~3 hours (large gels) at 200V.

2.2.6 Transfer of proteins from SDS-Polyacrylamide gels to solid support

For the purpose of Western blotting, proteins from SDS polyacrylamide gels were immobilised on nylon PVDF membrane (Amersham Biosciences) by transfer at 400mA for 45 minutes in 1×Towbin using the Biorad Mini-Protean II System.

2.2.7 Protein identification by matrix-assisted laser desorption–ionization/ time-of-flight-mass spectrometry (MALDI-TOF-MS) analysis.

Protein bands of interest were excised from SDS-PAGE gels (see section 2.2.5) and sent to the Edinburgh Protein Interaction Centre for analysis by peptide mass fingerprinting. Briefly, excised gel bands were subjected to in-gel digestion with trypsin. The resultant peptides were extracted from the gel pieces and peptide mass lists generated from the spectrum obtained by MALDI-TOF Mass Spectrometry (Applied Biosystems Voyager DE STR MALDI-TOF mass spectrometer). These masses were submitted to a database search engine such (SwissProt and Mascott) to identify a list of potential identities.

2.3 Mammalian cell culture

All mammalian cells were maintained at 37°C in a humidified incubator under 5% CO_2 and were generally passaged three times every week. Refer to table 2-12 for details of the mammalian cell-lines used in this work.

2.3.1 Adherent cells

Adherent cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v Serum Supreme, 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 2mM L-glutamine (Gibco). Sub-confluent cultures were trypsinised (trypsin-EDTA; 0.05% trypsin, 0.53M EDTA-4 Na) for ~3 minutes and the appropriate fraction of cells re-seeded in fresh growth medium.

Name	Derived from	Culture type	Supplements	Reference
A549	lung carcinomatous tissue	adherent		Giard, Aaronson et al. 1973
CHO-K1	Chinese Hamster Ovary (epithelial) derived as a subclone from the parental CHO cell line.	adherent	Non essential amino acids	Puck, Cieciura et al. 1958
COS-1	derived from the CV-1 cell line (kidney of a an African green monkey) by transformation with an mutant of SV40 which codes for wild type T antigen	adherent		Gluzman 1981
HELA	human cervical carcinoma	adherent	Non essential amino acids	Scherer, Syverton et al. 1953
MCF-7	adenocarcinoma of mammary gland (epithelial)	adherent		Brooks, Locke et al. 1973
293T	primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA.	adherent		Aiello, Guilfoyle et al. 1979
BJAB	Epstein-Barr virus-negative Burkitt's lymphoma	suspension		Clements, Klein et al. 1975; Steinitz and Klein 1975
K-562	multipotential, haematopoietic malignant cells from a patient with chronic myelogenous leukemia (CML)	suspension		Lozzio and Lozzio 1975
Mutu1	Epstein-Barr virus-positive Burkitt's lymphoma B-cell (clone 179)	suspension		Gregory, Rowe et al. 1990
sf9	Sf9 was cloned from the parent line, Sf21 (below)	semi-adherent		Vaughn, Goodwin et al. 1977
sf21	Pupal ovarian tissue of the fall armyworm, Spodoptera frugiperda	semi-adherent		Vaughn, Goodwin et al. 1977

Table 2-12 Cell lines used during this work.

2.3.2 Suspension cells

Cells were passaged by sub-culturing into fresh growth medium (RPMI 1640 supplemented with 10% v/v Serum Supreme, 100 U/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine).

2.3.3 Induction and assessment of apoptosis

Table 2-13 lists dose and incubation periods for the apoptosis-inducing agents used during this work. Cells were checked by microscopy under a bright field for gross morphological changes such as cytoplasmic shrinkage and membrane blebbing prior to making one or more of the assessments described in sections 2.3.3.1 to 2.3.3.3 inclusive.

2.3.3.1 Visualisation of condensed chromatin.

4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) or TO-PRO-3 was added, to cells to a final concentration of 0.25μg/ml or 3μM respectively. Samples were analysed by fluorescence microscopy. For quantitative analysis ~200 cells (fixed in 1% formaldehyde, BDH Chemicals) per sample were scored for apoptosis by the presence of condensed chromatin.

2.3.3.2 Annexin V detection of cell surface exposed phosphatidylserine.

2.5×10^5 cells were aliquoted to FACS tubes and washed in 1 ml of Annexin V binding-buffer (see buffers and solutions for composition) and pelleted before incubating on ice for 5 minutes with 1μl of fluorescein isothiocyanate (FITC)

labelled annexin V (diluted in 100µl annexin V binding buffer). Following incubation, cells were washed once more in annexin V binding buffer before assessment of cell-associated fluorescence by flow cytometry. In cases when membrane integrity was being assessed, propidium iodide (PI) was added immediately prior to analysis to a final concentration of 20µg/ml.

2.3.3.3 Light scattering properties

Flow cytometric analysis of forward (FS) and side-light-scatter (SS) was used to discriminate viable and apoptotic cells by virtue of their differing cell diameter and granularity (Dive, Gregory et al. 1992).

2.4 Insect cell culture

All insect cells were maintained at 27°C in a non-humidified ambient air-regulated incubator and were generally passaged three times every week by sub-culturing into fresh unsupplemented growth medium (Sf-900 II). Refer to Table 2-12 for details of the insect cell-lines used in this work.

2.5 Production of liposomes

These small lipid vesicles were prepared by sonication of an aqueous solution of phospholipids. The liposomes were composed of, L-α-phosphatidylcholine (PC), L-α-phosphatidylserine (PS), or a combination of the two at a ratio of 70% PC/30% PS. The phospholipids were added to glass tubes and the solvent evaporated under nitrogen. The resultant solid was mixed in DMEM and sonicated for approximately 20 minutes (sonicator bath) until the solid had disappeared and the solution had turned cloudy. Phospholipids were at a final concentration of 0.1mM.

Cell type	Induction method	Conditions	Assessment
Mutu 1	ionomycin	1μM, up to 16 hours	light scatter, AxV/PI, DAPI
	UV-B irradiation	200 mJ/cm^{2,} up to 16 hours	
CHO-K1	staurosporine	1μM, 5.5 hours	DAPI, AxV/PI
MCF-7	etoposide	100μM, 48-72hours	AxV/PI
K562	TRAIL (125ng/ml) and cyclohexamide (10μg/ml) 8 hours	8 hours	AxV/PI, DAPI
PMN's	aged the absence of serum	16-24 hours	AxV/PI

Table 2-13 Details of doses and incubation periods for the apoptosis-inducing agents used during this work. Refer to methods section x for detailed protocols for assessment of apoptosis. AxV; Annexin V, PI; Propidium Iodide, DAPI; 4',6-diamidine-2'-Pheny

Assay	Plate coating	Plate blocking	Antibody diluent
Standard antibody ELISAs	PBS, overnight, 4 degrees C	2%MPBS, 1 hour, RT	2%MPBS
Phage antibody ELISAs	PBS, overnight, 4 degrees C	2%MPBS, 1 hour, RT	10%NGS/2xPBS
Lipid/proteoliposome phage antibody ELISA	in DMEM, 45 min, 37 degrees C	4%BSA/PBS, 30 min, 37 degrees C	10%NGS/2xPBS

Table 2-14 Assay specific conditions used for ELISAs. PBS; phosphate buffered saline, NGS; Normal goat serum, 2%MPBS; 2% Skimmed milk powder in PBS.

2.6 Isolation of human peripheral blood monocytes and neutrophils.

Mononuclear cells were isolated under sterile conditions from peripheral blood of normal healthy human donors by density centrifugation on a Percoll® (Amersham Biosciences) gradient at room temperature. Sodium citrate was added to 120 ml of blood to a final concentration of 15.5 mM and centrifuged at 325g for 20 minutes with no brake. Half of the resulting supernatant (plasma) was removed and centrifuged at 2000g for 15 minutes to pellet the platelets. The resulting platelet-poor plasma (PPP) was reserved. Calcium chloride was added to the remaining platelet-rich plasma (PRP) to a final concentration of 40 mM before incubation at 37°C for 1 hour or until a tight clot had formed (reserved for monocyte culture). 5ml of 6% (w/v) dextran-500 and 20 ml of saline were added per 25 ml of plasma-poor blood in a 50ml conical polypropylene tube (Becton Dickinson). The solution was mixed by gentle inversion and incubated at room temperature for 30 minutes to allow sedimentation of red blood cells. Following sedimentation the upper white blood cell layer was removed and centrifuged for 10 minutes at 200g. The cell pellets were combined, resuspended in a total of 4ml PPP before dividing between two fresh 15ml polypropylene tubes. 42% and 51% solutions of Percoll® were prepared in PPP. A 2ml aliquot of the 42% Percoll® solution followed by 2ml aliquots 51% solution were used to underlay the white blood cell suspensions before centrifugation at 225g. Cells from the resulting monocyte and neutrophil layers were removed by pipette and washed twice in serum-free RPMI prior to use.

2.7 In vitro differentiation of monocytes to macrophages

Isolated monocytes were resuspended in Iscoves Modified Dulbeccos' Medium (IMDM) at 2×10^6 cells/ml. 200µl aliquots of cell suspension were added per well of a Teflon coated 4 well glass slide and incubated at 37°C to allow adherence of the monocytes to the glass surface. Following 1 hour of incubation the slides were washed with serum-free RPMI to remove non-adhering cells. The remaining cells were cultured for 7 to 8 days in IMDM supplemented with 10% autologous serum (PRP prepared as described in section 2.6) and penicillin/streptomycin at 100U/ml and 100µg/ml respectively. Slides were washed with serum-free RPMI and fresh growth medium replaced every second or third day.

2.8 In vitro differentiation of THP-1 cells to macrophages

200µl of THP-1 cells at density of 1.5×10^6/ml in complete DMEM (see section 2.3.1) containing dihydroxyvitamin D3 (VD3) (250nM), and phorbol 12-myristate 13-acetate (PMA) (100nM) were seeded onto wells of round 4 well Teflon coated slides. VD3 and PMA stimulated cells were allowed to incubate for ~60 hours prior to use.

2.9 Culture of mouse peritoneal macrophages

Peritoneal cells, harvested by lavage with ice-cold PBS, were cultured in complete DMEM supplemented with conditioned medium from mouse L-cell cultures (10% v/v) for 24 hours prior to use.

2.10 Macrophage interaction assays

Cultures of cell lines induced into apoptosis (feed cells) were washed twice with serum-free RPMI, by centrifugation at 1000rpm for 5 minutes before resuspending at $1x10^7$ cells/ml in serum-free RPMI containing 0.2% bovine serum albumin (BSA) (assay medium). Antibodies, inhibitors or control samples were diluted to twice the final required concentration in Eppendorf tubes using assay medium before adding an equal volume of feed cells at $1x10^7$/ml. Sample tubes were covered and inverted gently to mix. Differentiated THP-1cells, monocyte-derived macrophages, or mouse peritoneal macrophages were prepared by washing Teflon-coated slides in a bath of pre-warmed serum-free RPMI immediately before adding 200µl of the desired sample to each macrophage-containing well. Slides were incubated for 1 hour at 37°C, 5% CO_2 for interaction assays or 45 minutes at RT for binding assay before washing away unbound feed cells by dipping slides a total of 6 times in two baths of ice-cold PBS. The slides were fixed in methanol for 10 min at RT and air-dried. For scoring assays by light microscopy, cells were stained by immersing in diluted Jenner stain (BDH Chemicals) for 4.5min, washing in a bath of Jenner/Giemsa buffer (see appendix 2-1), and immersing in diluted Giemsa stain (BDH chemicals) for 9.5min. The slides were washed once more in a bath of Jenner/Giemsa buffer followed by distilled water. After air-drying the slides were mounted in DePeX (BDH Chemicals) prior to examination by light microscopy. The number of feed cells both phagocytosed and/or bound by macrophages was scored using previously established criteria (Flora and Gregory 1994) in duplicate wells for each sample with a minimum of 200 macrophages being assessed in each well.

2.11 Immunoglobulin based detection, measurement and characterisation techniques

2.11.1 Enzyme-linked immunosorbent assays (ELISAs)

Wash steps were carried out with PBS containing 0.05% Tween-20 (PBS-T) bu PBS alone for liposome ELISAs. In all cases peroxidase-conjugated antibodies were used in the final detection step. For analysis, optical densities of the assay solutions were read at 490nm using an Anthos lt 2 plate-reader (Labtech).

2.11.1.1 Plate preparation

Refer to table table 2-14 for details of assay-specific antigen concentrations and buffers. 100µl of diluted antigens were used to coat wells of microtitre plates. Samples containing protein antigens or LPS were incubated overnight at 4°C, liposome samples were incubated for 45 minutes at 37°C. Following incubation the solutions were tipped out before blocking any non-occupied binding sites on the plastic by adding 200µl of blocking buffer (see table 2-14) and incubating for 1 hour at room temperature. Wells were emptied and washed 3 times before the further addition of reagents.

2.11.1.2 Assay procedures

To make an assessment of non-specific signals, wells containing all possible combinations of coating antigens/target capture antibodies and detection antibodies were included. During any given step an equal volume of reagent diluent was added to wells which did not require sample/antibody.

2.11.1.2.1 Direct ELISA

100µl of diluted primary antibodies were added per well of prepared plates and incubated for 1hour at room temperature. Wells were emptied and washed 3 times before adding 100µl of diluted secondary antibody to the appropriate wells and incubating for a further 1hour at room temperature. On occasions when a tertiary antibody step was required for detection, wells were emptied and washed 3 times before adding the appropriate diluted antibody for an additional incubation of 1hour at room temperature. Prior to completion of the final incubation step the o-phenylenediamine-based substrate, SIGMA-FAST™ (Sigma) was prepared according to manufacturers' instructions. Following 4 more washes, 100µl of substrate was added per well. Once an adequate colour change was seen the reaction was stopped by the addition of 50µl 2.5M HCl per well.

2.11.1.2.2 Antigen-capture ELISA

100µl of diluted antigen-containing sample were added per well of prepared plates and incubated for 2 hours at room temperature. Wells were emptied and washed 3 times before adding 100µl of diluted primary detection antibody to the appropriate wells and incubating for a further 1hour at room temperature. Following incubation, wells were emptied and washed 3 times before adding 100µl of secondary detection antibody to the appropriate wells and incubating for a further 1hour at room temperature. On occasions when a tertiary antibody step was required for detection wells were emptied and washed 3 times before adding the appropriate diluted antibody for an additional incubation of 1hour at room temperature. Prior to completion of the final incubation step the o-phenylenediamine-based substrate,

SIGMA-FAST™ (Sigma) was prepared according to manufacturers' instructions. Following a 4 more washes, 100μl of substrate was added per well. Once an adequate colour change was seen the reaction was stopped by the addition of 50μl 2.5M HCl per well.

2.11.2 Immunofluorescence for flow cytometry

5% normal goat serum (Harlan Sera-Labs) in PBS (NGS/PBS) or 1% BSA was used for diluting antibodies. All wash steps were carried out using NGS/PBS or 1% BSA in PBS by centrifugation at 225g for 5 minutes at 4°C then discarding the supernatant. Cultured cells were harvested by centrifugation at 225g for 5 minutes at 4°C then discarding the supernatant. Cells were washed using a volume equal to that harvested from before resuspending at a density of 5×10^{6}/ml in wash buffer. 50μl aliquots of cell suspension were added per well of a 96-well round-bottomed cell culture plate. A volume of 200μl was used for all subsequent washes. Cells were incubated with 50 μl of diluted primary antibody for 30 minutes at 4°C. After washing twice, 50 μl of secondary antibody was added and incubated for a further 15-30 minutes at 4°C. On occasions when a tertiary antibody step was required two more washes were carried out before incubating cells with 50μl of diluted antibody for 15-30 minutes. Two final washes were performed before using 200μl of 1% formaldehyde in NGS/PBS to transfer cells to FACS tubes containing an additional 300μl of 1% formaldehyde in NGS/PBS.

2.11.3 Immunofluorescence analysis by confocal laser scanning microscopy

200µl cells in complete DMEM at density of 1.5×10^6 were seeded on wells of round 4 well Teflon coated slides 24 hours prior to analysis. Cells were washed once with PBS and fixed at RT in 3% formaldehyde/5% sucrose/0.2% Triton X-100 in PBS for 2 minutes then for I hour in the same buffer without Triton X-100. After 3 washes in PBS excess free aldehyde groups were quenched with 50mM NH_4Cl in PBS for 15 minutess. Cells were washed 3 times in PBS before incubating with primary antibodies diluted in NGS/PBS 20-30 minutes at 4°C. After washing cells in PBS, bound antibodies were revealed by incubating with conjugated secondary antibodies diluted in NGS/PBS for 20-30 minutes in the dark at 4°C. After 3 washes in PBS and one wash in dH_2O samples were allowed to air dry before mounting in Mowiol with 10 mm round coverslips and viewed using confocal laser scanning microscope (CLSM) systems (Leica and Zeiss) coupled with Ti:Sapphire lasers.

2.11.4 Immunological detection of immobilised proteins

2.11.4.1 Western blotting on PVDF membrane

All wash steps were carried out using 10ml PBS with 100mM extra NaCl (PBS+) for 5-10 minutes at room temperature. All antibodies were diluted in 5% Skimmed Milk Powder in PBS (5%MPBS). A volume of 4ml was typically used per membrane. PVDF membranes were blocked overnight in 5%MPBS milk at 4°C and washed three times before probing with antibodies. Blocked membranes were incubated with primary antibodies for 2 hours at 4°C, followed by three washes and incubation with peroxidase-conjugated secondary antibody for 1 hour at room temperature.

Following three final wash steps, bound HRP-conjugated antibodies were detected using one of two methods.

2.11.4.1.1 a) Chemiluminescence

Membranes were immersed in ECL reagents (Amersham Pharmacia Biotech) following manufacturers' protocol before dark exposure to autoradiography film for variable lengths of time and developing in an automated developer (Konica SRX-101A).

2.11.4.1.2 b) Colourimetric

A solution containing 30mg of 4-chloronapthol (4-CN) in 10ml of absolute methanol at -20°C was prepared and added to 50 ml of ice-cold TBS. 30µl of H_2O_2 was added immediately prior to incubation of membranes in the solution. When the desired band(s) could be seen the reaction was stopped by washing membranes in dH_2O.

2.11.4.2 Dot blot quantification of M13 coat protein

All wash steps were carried out using 10ml 5% TBS containing 0.05% Tween-20 (TBST) for 5-10 minutes at room temperature. The concentration of phage particles in samples was determined by titration. Two-fold serial dilutions of test samples or standard were prepared in TBS. Diluted samples were pipetted directly onto pre-equilibrated PVDF membrane and allowed to adsorb for ~1 minute. Membranes were blocked for 1hour at RT with 5% milk powder/TBS (5%MTBS). Blocked membranes were washed three times before incubating with peroxidase-conjugated α-M13 monoclonal antibody for 1 hour at room temperature. Following three final

wash steps, bound detection antibodies were analysed by chemiluminescence as described above.

2.11.4.3 Immunopreciptation

All wash steps were performed by placing the incubation vessels next to a magnetic separator for 1 minute before removing the supernatant from the beads with a pipette. All wash steps were performed using PBS containing 1% v/v Trition-X-100 and 0.7% w/v deoxycholic acid.

2.11.4.3.1 Bead coating

16μl of resuspended magnetic α-mouse IgG-coated beads (Sigma) were equilibrated by washing three times before incubating with 15μg the desired antibodies in a total of 0.5 ml in PBS for 30 minutes at 4°C with rotation.

2.11.4.3.2 Antigen capture

Cell lysates were prepared as described in section 2.2.2.1 with the exception that PBS containing 1% v/v Trition-X-100 and 0.7% w/v deoxycholic acid was used as a solubilisation buffer allowing 1×10^7 cells per bead sample. Cell lysates were pre-cleared by incubation with uncoated beads for 45 minutes at 4°C with rotation. Coated beads were allowed to incubate with pre-cleared cell lysates for 4 hours at 4°C with rotation. Following incubation beads were washed three times before adding to Laemli sample buffer in preparation for analysis by SDS-PAGE as described in section 2.2.5.

2.12 Phage antibody display techniques

2.12.1 The bacteriophage library

The phage display library used for the work described in this thesis was constructed from 49 previously cloned, human germline immunoglobulin heavy-chain variable (V_H) genes fused to a heavy-chain joining segment 4 and partly randomised complementarity-determining region 3 (CDR3) sequences of 6 to 15 amino acid residues in length. The V_H segments were cloned into seven vectors containing light chains encoded by members of the $V_\kappa 1$ to $V_\kappa 4$ and $V_\lambda 1$ to $V_\lambda 3$ gene families. Individual libraries were then combined to form a single library of approximately $3.6x10^8$ clones(Dekruif, Boel et al. 1995).

2.12.2 Amplification of phage antibodies

2.12.2.1 Large scale

50ml of LB with 5% glucose (Sigma) and 100μg/ml of ampicillin (Sigma) were inoculated with phage infected TG1 cells and grown with shaking at 37°C to an OD_{600nm} of 0.4-0.5 before sub-culturing 5ml into 50ml of fresh LB with 5% glucose and 100μg/ml ampicillin and adding 5×10^{10} plaque forming units (pfu) of VCS-M13 helper phage. The cultures were incubated statically at 37°C for 45 minutes followed by 45 minutes with shaking before centrifugation at 2000 g for 20 minutes. The resulting bacterial pellet was resuspended in 250 ml LB with 100μg/ml ampicillin and 25μg/ml of kanamycin (Sigma) and grown overnight with shaking at 30°C. Phage particles were purified from the bacterial culture supernatant by two successive precipitation rounds using polyethylene glycol (PEG_{8000}) (Sigma) as

follows; overnight cultures were centrifuged at 6000g for 10 minutes at 4°C and the supernatant incubated in a fresh vessel on ice for 1 hour with 1/5 volume of 20% (w/v) PEG $_{8000}$ in NaCl (2.5M) (PEG/NaCl). The precipitate was harvested by centrifugation at 2000g for 30 minutes, resuspended in 40ml PBS and incubated on ice for 30 minutes with 1/5 volume PEG/NaCl. The precipitated phage particles were again harvested by centrifugation at 2000g for 30 minutes before resuspending in 3.6 ml of 1% (w/v) BSA in PBS. Resuspended phage solutions were passed through a 0.45μm filter for sterilisation before storing at 4°C.

2.12.2.2 Small scale growth of monoclonal phage antibodies

Individual colonies from agar plates were inoculated into wells of 96-well microtitre plates containing 100µl LB with 5% glucose and 100µg/ml of amp. The plates were incubated overnight at 37°C, with shaking. The following day 10µl of the cultures were inoculated into wells of freshly prepared plates containing 100µl of LB with 5% glu and 100µg/ml of ampicillin. Following 2 hours of incubation at 37°C with rotation, 10µl of LB containing 10^9 pfu of VCS-M13 helper phage was added to each well. Plates were incubated statically at 37°C for 1hour before centrifugation at 2000g for 20 minutes to pellet the infected bacteria. The supernatant was discarded and the bacteria resuspended in 250µl of LB with 100µg/ml ampicillin and 25µg/ml kanamycin for incubation overnight at 30°C.

2.12.3 Growth and purification of the library

2.12.3.1 Purification by PEG precipitation

The complete library supplied from Prof. Ton Logtenberg was amplified from glycerol stocks of 12 sub-libraries. Each sub-library was amplified as described in section 2.12.2.1 (Large scale amplification of Phage Antibodies) before pooling and storage at 4°C.

2.12.3.2 Phage purification on caesium chloride gradients

This method, adapted from Smith and Scott (Smith and Scott 1993) is effective in removing residual PEG, nucleases, proteases and other contaminants from PEG-purified phage and is recommended for long-term storage.

4.83g of caesium chloride (CsCl) was resuspended in10ml of freshly grown and PEG-purified phage library (from section 2.12.3.1). TBS was added to the resultant solution to a final weight of 10.75g giving a 31% (w/w) solution of CsCl with a density of 1.3g/ml. The solution was aliquoted to 12-ml polyallomer tubes and centrifuged at 58,000 rpm for 20 hours at 4°C using a Beckman 70.1 Ti rotor. Following centrifugation the faint, bluish upper phage bands were removed from each tube using a sterile glass pipette combined and resuspended in 26ml of TBS before centrifugation at 48,000 rpm for 4 hours at 4°C using a Beckman 50.2 Ti rotor. The final pellet was resuspended in 12ml TBS containing 0.1% (w/v) gelatin, 0.02% NaN_3 and stored at 4°C.

2.12.4 Selection of monoclonal phage antibodies

2.12.4.1 Whole cell-based selections

2.12.4.1.1 Selection on K562 cells

All washes were carried out using 5 ml of 1% BSA in PBS by centrifugation at 1000 rpm for 3-5 minutes at 4°C then discarding the supernatant.

1 ml of PEG-purified phage particles (~5×10^{11} c.f.u.s) blocked in 2.25 ml of 4% non fat milk powder in PBS (4% MPBS) for 15 minutes at RT were incubated with $7.5\text{x}10^6$ of pre-washed cells resuspended in 3 ml of 4%MPBS for the required time at 4°C with gentle inversion. After incubation the cells were washed to remove non-binding phage antibodies from the cells. The final wash was carried out in 1 ml of 1%BSA in PBS before eluting phage from the cells by resuspending in 100μl of PBS and 150μl of 76mM citric acid (pH 2.5) in PBS and incubating for 5 minutes at RT. The cells were pelleted and the phage-containing supernatant transferred to a fresh Eppendorf with 200μl of 1M Tris HCl (pH 7.4) added for neutralisation. Eluted phages were used to infect *E. coli* strain TG1 as described in section 2.12.5.

2.12.4.1.2 Flow cytometry whole cell-sorting-based selections

All washes were carried out using 5 ml of 1% BSA in PBS by centrifugation at 1000 rpm for 3-5 minutes at 4°C then discarding the supernatant.

$1\text{x}10^7$ pre-washed viable ICAM-3 negative Mutu I cells (~10% apoptotic as assessed by Annexin V/PI staining) were added to $3\text{x}10^5$ pre-washed ionomycin-induced ICAM-3 positive Mutu I cells (~80% apoptotic as assessed by Annexin V/PI staining) in a total of 2ml of 4% BSA/PBS (hence a total of ~25% apoptotic cells in

the entire population). Cells were added to 0.5 ml of CsCl-purified phage particles (~2.5×10^{11} c.f.u.s) that had previously been blocked in 0.6ml of 4%BSA/PBS for 15 minutes at RT and incubated for 20 minutes on ice, with occasional mixing by inversion. After incubation the cells were washed once to remove non-binding phage antibodies before using light-scattering properties as a criteria for collecting apoptotic cells by flow cytometry (see section 2.3.3.3). Phages were eluted from the cells of the sort sample by resuspending in 100μl of PBS and 150μl of 76mM citric acid (pH 2.5) in PBS and incubating for 5 minutes at RT. The cells were pelleted and the phage-containing supernatant transferred to a fresh Eppendorf with 200μl of 1M Tris HCl (pH 7.4) added for neutralisation. Eluted phages were used to infect *E. coli* strain TG1 as described in section 2.12.15.

Target antigen	Solid support	Selection conditions		Elution	Post-elution depletion	No. of selections prior to screening	Results section ref
		Blocking Steps					
		Library	Target				
ICAM3-Fc	Immunotube	2%MPBS 15 min RT	2%MPBS 2 hours RT	Glycine pH2.2	none used	3 rounds positive selection	4.1.3.1
Phosphatidylserine	Immunotube	4%BSA/PBS 15 min RT	4%BSA/PBS 1hour, 37 degrees C	First round glycine pH2.2. Subsequent rounds PS liposomes or whole apoptotic mutu1 cells	PC coated immunotubes	2-3 rounds positve selection, specific elution	4.2.2
ICAM-3-Fc	Protein A coated beads	4%BSA/PBS 15 min RT	20%NGS/PBS 30 minutes, RT	Glycine pH2.2	none used	3 rounds positive selection	4.1.3.1
His-tagged ICAM-3	Nickel-charged beads	4%BSA/PBS 15 min RT	none used	imidazole	none used	1-3 rounds positive selection, elution with imidazole	4.1.3.2
Apoptotic cell-derived proteoliposomes	Immunotubes	complete RPMI + 5%BSA followed by depeletion for 1/2hr hour on viable CHO cell monolayer. (BSA blocked) and 1/2hr on complete medium coated immunotube	complete RPMI + 5%BSA	50mM Glycine (pH2.2) - Neutralisation with Tris-HCl (pH 8.8)	Immunotubes coated with (a) BSA then (b)complete RPMI.	1-2 rounds	4.3.2

Table 2-15. Conditions used for individual phage antibody selections. MPBS; skimmed milk powder in PBS, NGS; Normal Goat Serum, RT; room temperature.

2.12.4.2 Selections on immunotubes

2.12.4.2.1 Coating of immunotubes

For each target antigen optimal coating concentrations were determined empirically by titration in an ELISA. 4 ml of diluted antigen was added to immunotubes (Nunc) and incubated for the required time using identical buffers to those used for determining optimal coating concentration. Refer to table 2-15 for selection-specific buffers and incubation periods. Following incubation, tubes were washed 3 times with PBS then filled with the appropriate blocking buffer and incubated for 0.5-2 hours at RT (see 2-15). Tubes were washed 3 times with PBS before the addition of phage library for selection.

2.12.4.2.2 Selection

1ml of purified phage library (~5×10^{11} c.f.u.s) in a total volume of 3.5 ml blocking buffer was added to pre-blocked immunotubes and incubated with rotation for 30 minutes (for ICAM-3-Fc selections tubes were left to stand for an additional 90 minutes at RT). Following incubation, the tubes were emptied and unbound phages were removed by washing as follows;

For first-round selections tubes were washed 10 times with PBST (PBS only for liposome selections), then 10 times with PBS. For second and subsequent rounds of selection, tubes were washed 20 times with PBST (PBS only for liposome selections), and 20 times with PBS.

2.12.4.3 Selections using His-tagged antigen bound to nickel-charged beads

All wash steps were performed by placing the incubation vessels (1.5ml Eppendorf tubes for selections or 96-well plates for bead-coating-optimisation) next to a magnetic separator for 1 minute before removing the supernatant from the beads with a pipette. 50 mM sodium phosphate, pH 8.0, 0.3 M NaCl with 0.005% Tween-20 was used for all wash steps (1ml for selections, 150µl for bead-coating-optimisation).

2.12.4.3.1 Bead coating

Nickel-nitrilotriacetic acid magnetic agarose beads (nickel-charged beads) (Qiagen) were washed twice before incubating with the desired volume of His-tagged ICAM-3 for 30 minutes at RT with rotation (Eppendorf tubes) or gentle rocking (96-well plates).

Beads were washed three times before the addition of phage library for selection.

2.12.4.3.2 Selection

1ml of purified phage library (~5×10^{11} c.f.u.s) in a total volume of 3.5 ml blocking buffer was added to pre-coated beads and incubated with rotation for up to 30 minutes. Following incubation, the beads were washed 10 times with PBST and a further 10 times with PBS. Refer to table 2-15 for antigen specific selection and elution conditions.

2.12.5 Infection of *E. coli* with phage particles.

Cultures of E. coli TG1 were grown to an OD_{600nm} of 0.4-0.5 at 37°C in LB. Solutions of phages were added to 2ml of the log phase *E. Coli*, and pre-warmed LB in a total volume of10ml and left to incubate statically for 1hour at 37°C. The culture was then centrifuged at 2000g for 15 minutes at RT. The bacterial cell pellet was resuspended in 1ml LB and serially diluted aliquots spread onto agar plates containing LB with 100μg/ml amp and 5% glu for incubation overnight at 37°C.

The following day, colonies from the plates were counted and used for one of three purposes. For the purpose of screening individual colonies were picked for monoclonal production as described in section 2.12.2.2. Alternatively colonies were scraped into a total of 2ml of LB and aliquots used either to make a polyclonal stock in 15%glycerol for storage at -80°C or used to inoculate 50ml of LB with 5% glucose and 100μg/ml ampicillin for large-scale amplification and purification for further rounds of selection.

2.12.6 Cell-based screening of phage antibodies.

Immunofluorescence staining of cells was carried out as described in section 2.11.2 for assessment by flow cytometry or microscopy with the exception that phage antibodies were diluted in 10% NGS in 2xPBS as opposed to instead of 5%NGS/PBS.

2.12.7 Screening of phage by ELISA

ELISAs were carried out as described in section 2.11.1 using the conditions shown in table 2-14.

3 Selecting for Apoptotic-Cell-Surface Markers from a Phage-Displayed Antibody Library.

The molecular identities of the endogenous ligands of apoptotic cells that can be used by phagocytes for recognition and engulfment are poorly understood. The work described in following sections specifically attempts to profile novel surface molecules appearing during apoptosis using a library of antibody fragments displayed on the surface of bacteriophage particles.

3.1 Results Chapter 1 - Using Whole Cells to Select for Specific Markers of Apoptosis

The work described in this section was targeted towards the use of phage display and whole cells to select antibody fragments with specificity towards Intercellular Adhesion Molecule-3 (ICAM-3), a cell-surface-expressed molecule implicated in apoptotic-cell recognition. However the screening strategies for testing the binding specificity of monoclonal phage were designed to allow identification of additional markers of apoptosis.

3.1.1 Experimental design

3.1.1.1 Practical considerations

Using the surface of whole cells as a source of target antigen for selection has several inherent problems. The cell surface is a highly heterogeneous fabric with most individual epitopes being expressed at relatively low concentration. Therefore, of those antibodies selected which are specific to the cell surface, only a minority are likely to be specific to molecules or certain epitopes distinctly found on the apoptotic cell.

Selecting on apoptotic cells imposes additional challenges. As will become apparent from the discussion below, an unavoidable situation arises in which selection of phage antibodies on cells of the most appropriate physiologically relevant stage of death (where they are recognised and ingested by phagocytes) requires the cells to be in a fragile state. For selection purposes this is inconvenient, as a cell that has lost its membrane integrity will provide a wealth of undesired intracellular antigens as potential phage targets. Moreover, its fragility at this stage does not lend the apoptotic cell to the rigorous washing conditions necessary for the recovery of only the highest affinity, specific antibody fragments.

3.1.1.2 Choice of selection strategy

For planning the selections described in the following section, the results of former work using the phage library employed for these studies were considered. A phage antibody against CD14, clone Ce3, had been previously isolated from the library used in the present studies by successive rounds of negative and positive selection on

CD14 negative and positive cells respectively (Pierce 2000). However, attempts by Pierce to extend these studies by following a similar strategy for cell-based selections failed to enrich for the desired binders. Furthermore, initial work carried out for this thesis in which a number of single-round positive selections were carried out on several different populations of apoptotic cells detected very few phage clones at the screening stage capable of binding to the cell type used for selection, either in viable or apoptotic states (data not shown). The reasons behind the lack of cell reactivity were not known, and there was no way of determining whether the particular conditions used during a selection had successfully enriched for cell-binding clones before launching large-scale screening exercises. Therefore it was necessary to set up a system for monitoring the effectiveness of: -

(a) the conditions used during a selection (i.e. a means to evaluate the relative number of phage particles obtained following a selection).

(b) the enrichment efficiency of sought-after phage. (i.e. a means of evaluating how well clones that bind to the target were separated from clones that do not).

In order to gauge the effect of the different selection conditions being tested (point a), the number of phage clones returning from a selection was calculated by titrating aliquots of phage-infected *E. coli* TG1 on bacterial culture plates and counting drug-resistant colony forming units (c.f.u.). To determine the efficiency of a given selection strategy (point b), polyclonal phage preparations were made after each selection and analysed for binding to cells by flow cytometry (i.e. if a significant proportion of the clones obtained from a selection are cell-reactive, a polyclonal mixture of such phage should consequently stain cells).

Additional features included in the experimental design were: -

(i) raising the target antigen (ICAM-3) concentration present on each cell by using the K562 cell line overexpressing ICAM-3 (K562/ICAM-3). However it was understood that such high levels of expression in combination with the asynchronous nature of apoptosis amongst *in vitro*-induced populations might not have resulted in all ICAM-3 being present in the "apoptotic form".

(ii) optimisation of selection conditions using viable K562/ICAM-3 cells prior to transfer of the system to apoptotic cells in order to obtain a pool of cell-binding phage amenable to analysis for specificity towards ICAM-3 in its apoptotic or viable form.

3.1.2 Results

3.1.2.1 Assessing the effects of incubation time and wash stringency on the recovery of phage particles from selections

The enrichment efficiency of the desired phage from a selection is dependent on a number of factors. These include concentration of target antigen and phage, temperature, buffer pH and ionic strength, incubation time and severity of washing procedures. The decision was made to test the effect of varying just two selection conditions. Firstly, the effect of washing severity was tested as previous work using this library for selecting on cells had suggested that washing cells more rigorously after the selection decreases the number of non-specific phage returning (Sarah Pierce, Andrew Devitt personal communication). Secondly, the effect of decreasing incubation periods from those previously used during a selection was assessed. It was hypothesised that shorter incubations would allow selection of only the highest

affinity antibodies and reduce non-specific binding of phage which is a problematic phenomenon known to occur during selections (Dekruif, Boel et al. 1995). Following each selection, recovered phage were used to infect *Escherichia coli* TG1 and colony forming units (c.f.u.) were used as a device for measuring phage number returning from each selection to enable a comparison to be made between selections of differing stringency.

Preliminary studies showed there to be a clear reduction in the number of phage particles recovered as selection stringency increased. However no selection of cell-binding phages was seen following single rounds of positive selection (data not shown). As a result a decision was made to try several rounds of positive selection in combination with more stringent conditions.

3.1.3Enrichment of cell binding phage using K562 cells transfected with ICAM-3

A marked reduction in the number of eluted phage clones was seen from selections carried out with 'harsh' washing (8 wash steps with vigorous mixing) compared to selections carried out with 'gentle' washing (2 wash steps with gentle mixing) (Figure 3-1 panel A). There were slightly, but consistently fewer clones returning from selections with 2-minute incubation periods compared to 1-hour (Figure 3-1 panel B). The relative difference in c.f.u./ml between panels A and B of figure 3-1 is a reflection of adding a different number of phage particles at the beginning of each selection for the two experiments. For all cell-based selections subsequent to those presented in panel B of figure 3-1 a constant number of phage particles and cells were used (see materials and methods section 2.12.3.1). Screening of individual

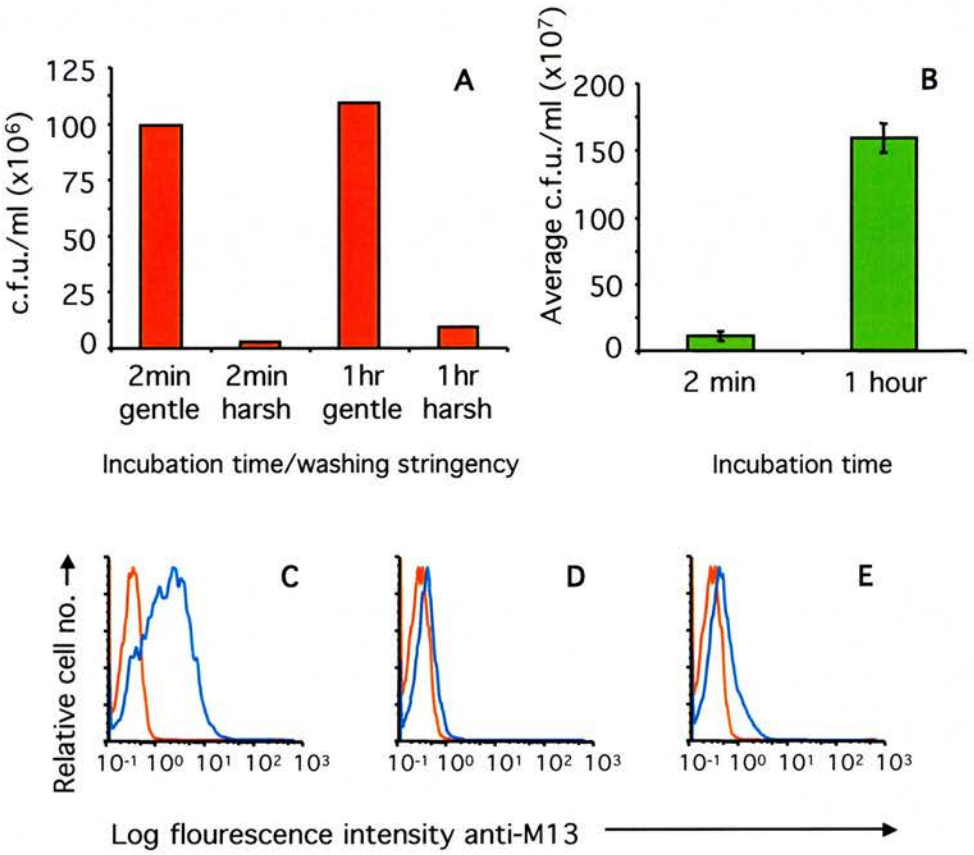


Figure 3-1. Results from single rounds of selection of phage antibodies on viable K562/ICAM-3 cells.

A,B; Phage were titred by infection of eluted particles into *Escherichia coli* TG1 and plating onto ampicillin-containing agar plates. **A** Selections were performed with short (2 min) or long (1 hour) incubations and the cells subsequently washed with differing severities (gentle or harsh). **B** Example of the difference in numbers of phage returning from selections with different incubation periods using harsh washes. Representative of 3 similar experiments. For panel B data points represent the mean +/- SD for duplicate titrations. c.f.u.; colony-forming units.

C-E; Phage antibodies were analysed for the ability to bind viable K562/CD14 cells (panel C) or viable K562/ICAM-3 cells (panels D&E) by flow cytometry. **C** Binding of phage antibody clone Ce3 (anti-CD14) to K562/CD14 cells was included during screening as a positive control for cell-staining. **D** Polyclonal phage antibodies prepared from the single-round selection on viable K562 cells using a 1-hour incubation period followed by harsh washing. **E** Example of a monoclonal phage antibody isolated from a single round of selection on viable K562 cells using a 1-hour incubation period followed by harsh washing. Red histograms represent control staining with mouse anti-M13 and goat anti-mouse-FITC antibodies.

phage clones from the selections referred to in figure 3-1 panels A and B identified no strongly-binding phage antibodies (Figure 3-1 panel E) and supernatants from polyclonal phage antibody preparations of the selections did not produce a significant signal when used to stain the same cells as those used for selection (Figure 3-1 panel D). It appeared that, despite more stringent selection conditions, phages were not being enriched usefully. With this in mind, the decision was made to return to multiple rounds of selection in an attempt to gain further enrichment. Four rounds of selection were carried out on viable K562/ICAM-3 cells with both 2-minute and 1hour incubation times. Two main points regarding the profile obtained from titrating the number of c.f.u.s returning from each selection are worth highlighting. Firstly, as was observed for the single-round selections mentioned previously, consistently fewer colonies returned from selections carried out using a 2-minute incubation period compared to a 1-hour incubation period. Secondly, the profile showed a similar trend for both incubation times, namely a slight decrease after the second round of selection followed by a sharp increase for the third round and an apparent levelling off after the fourth round. The assumption that a plateau is reached by round four is supported by the number of c.f.u. for a 2-minute incubation period "catching up" with the number of c.f.u. for a 1-hour incubation period (figure 3-2 panel A, B).

This trend could be explained by the persistence of non-specifically-selected phage in the sub-library following the first round of selection that are lost during the second round. After the third round, the increase in titre can be explained by duplication of the same clones during the amplification step between selections. The polyclonal

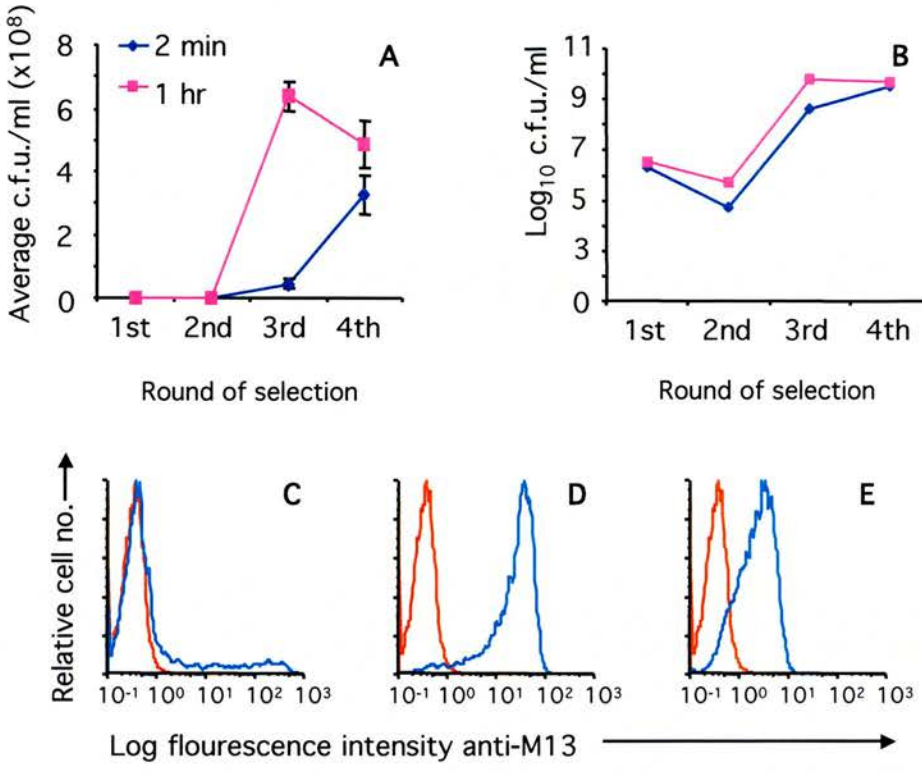


Figure 3-2. Results from multiple rounds of selection of phage antibodies on viable K562/ICAM-3 cells.

A,B; Profiles of phage returning from 4 successive rounds of selection (panel B displays the same data as A using a $\log_{10}$ scale for c.f.u./ml). Phage were titred by infection of eluted particles into *Escherichia coli* TG1 and plating onto ampicillin-containing agar plates. Data points represent the mean +/- SD for duplicate titrations.

C-E; Phage antibodies were analysed for the ability to bind viable K562/ICAM-3 cells by flow cytometry. **C** Polyclonal phage antibodies prepared from the 1st of 4 rounds of selection on viable K562 cells. **D** Polyclonal phage antibodies prepared from the 3rd of 4 rounds of selection on viable K562 cells. **E** Example of a monoclonal phage antibody from the 3rd round of selection on viable K562/ICAM-3 cells that demonstrated cell binding.The histograms shown in panels D-E are example of phage prepared from selections carried out with 2-minute incubations, however similar results were obtained with phage from selections using 1-hour incubations. Red histograms represent control staining with mouse anti-M13 and goat anti-mouse-FITC antibodies.

c.f.u.; colony-forming units

staining profile supported this explanation as there was no significant signal after the first round (Figure 3-2 panel C), a diffuse signal after the second round (suggesting the presence of high, low and non-specific phage), followed by a strong, clean signal by the third round (Figure 3-2 panel D) that did not strengthen after round four (data not shown for rounds two and four). 40 monoclonal phage antibodies from each of the third rounds (2-minutes and 1-hour incubation periods) were screened for their ability to bind K562/ICAM-3 cells. Of these, 8 clones from selections with 2-minute incubations and 9 clones from selections with 1-hour incubations demonstrated cell binding (example shown in figure 3-2 panel E).

3.1.4 Selection and screening of phage using apoptotic ICAM-3-transfected cells.

Following the success of the selections on viable cells, the same selection system was applied using apoptotic cells. Whilst appreciating that apoptotic-target cells may behave differently to their viable counterparts in this system, a 2-minute incubation time was chosen as there was no significant difference between the recovery of high affinity clones using this short length incubation than from the selections with 1-hour incubations for viable cell selections (8 and 9 clones respectively out of 40 tested for each incubation period). A shorter incubation would also help minimise the numbers of fragile apoptotic cells becoming permeabilised. Two cell types over-expressing ICAM-3 were chosen as apoptotic targets for phage selection. These were K562/ICAM-3 as used in the optimisation stage, and in addition CHO/ICAM-3 (see materials and methods section 2.3).

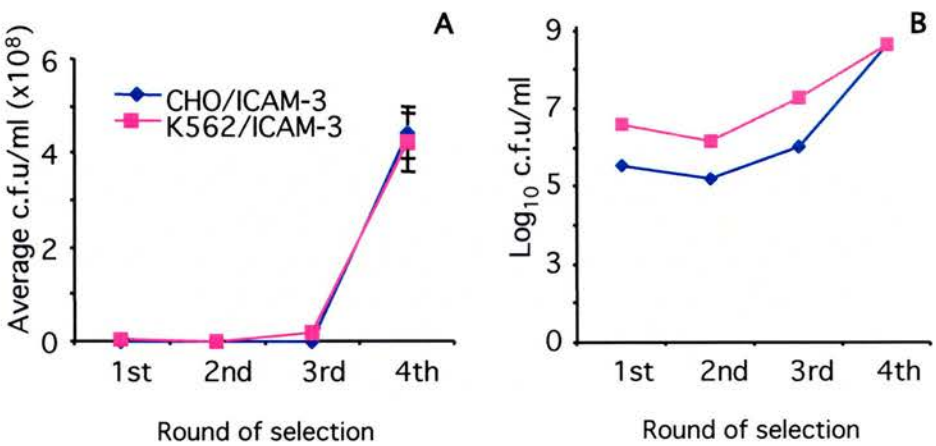


Figure 3-3. Results of selection of phage antibodies on apoptosis-induced cells. Phage were titrated by infection of eluted particles from each round of selection into *Escherichia coli* TG1 and plating onto ampicillin-containing agar plates. Selections on apoptotic CHO/ICAM-3 and K562/ICAM-3 cells followed the same trend as selections on viable K562/ICAM-3 cells. (A and B; linear and $\log_{10}$ scale representation of data respectively). Data points represent the mean +/- SD for duplicate titrations.

Four rounds of selection were carried out on each cell type. The profile for the number of c.f.u. returning from successive rounds matched that of viable cells. However the total number of colonies was slightly lower by the 4th round compared with viable cell selections (figure 3-3) despite using a fixed number of cells in each case. This may have been due to a lower amount of antigen being present on the apoptotic cell. In addition, the cell pellets were observed to decrease in size during the sequential wash steps, which apart from a continuation of apoptosis-associated cell-shrinkage (Benson, Heer et al. 1996), could have been due to the cells beginning to disintegrate (discussed in more detail, section 5.3).

The staining profile of polyclonal phage preparations was tested on various apoptosis induced and viable cell lines (figure 3-4). The polyclonal phage prepared from the third round of selection on CHO/ICAM-3 did not stain any cell line significantly (data not shown). The polyclonal phage antibodies prepared from the selections on K562/ICAM-3 cells stained viable and to a lesser extent apoptotic K562/ICAM-3 cells. The same preparation also stained viable K562/vector cells (ICAM-3 negative cell line) and unrelated Mutu I BL cells (figure 3-4 panels C-F). This suggests that the majority of selected phage were to antigens common to both viable and apoptotic cells. However, the possibility that a minority of clones amongst the selected population were specific for epitopes exclusively found on apoptotic-cell surfaces led to analysis of binding specificity of individual clones.

The approach taken for screening was to test each phage clone on either ICAM-3 positive or negative cells that were either induced into apoptosis or left untreated, thus allowing identification of phage specific to either "viable ICAM-3", "apoptotic ICAM-3", "generic ICAM-3"(clones which bind to ICAM-3 on viable and apoptotic

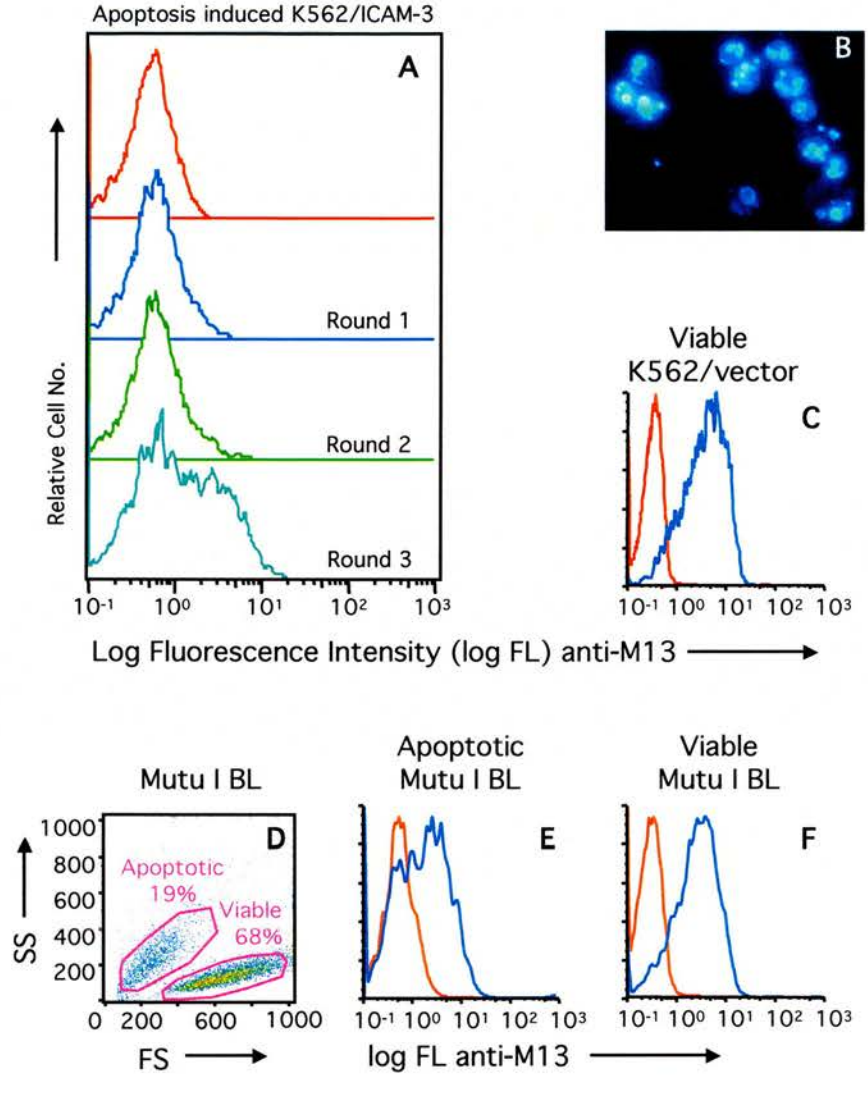


Figure 3-4. Binding of phage antibodies to various cell lines. Polyclonal phage antibodies prepared from selections on apoptosis-induced K562/ICAM-3 cells were analysed for their ability to bind various cell lines by flow cytometry. **A** Polyclonal phage antibodies prepared from the 1st, 2nd and 3rd round of selection on induced K562/ICAM-3 cells. **B** A combination of TRAIL and CHX induces classic apoptotic nuclear morphology in K562 cells as visualised by staining with DAPI. **C,E,F** Polyclonal phage antibodies prepared from the 3rd round of selection on viable K562/vector, induced Mutu I BL and viable Mutu I BL cell lines respectively. **D** Discrimination of viable and apoptotic Mutu I BL cells by measurement of forward (FS) and side (SS) light scatter. Red histograms represent control staining with mouse anti-M13 and goat anti-mouse-FITC antibodies.

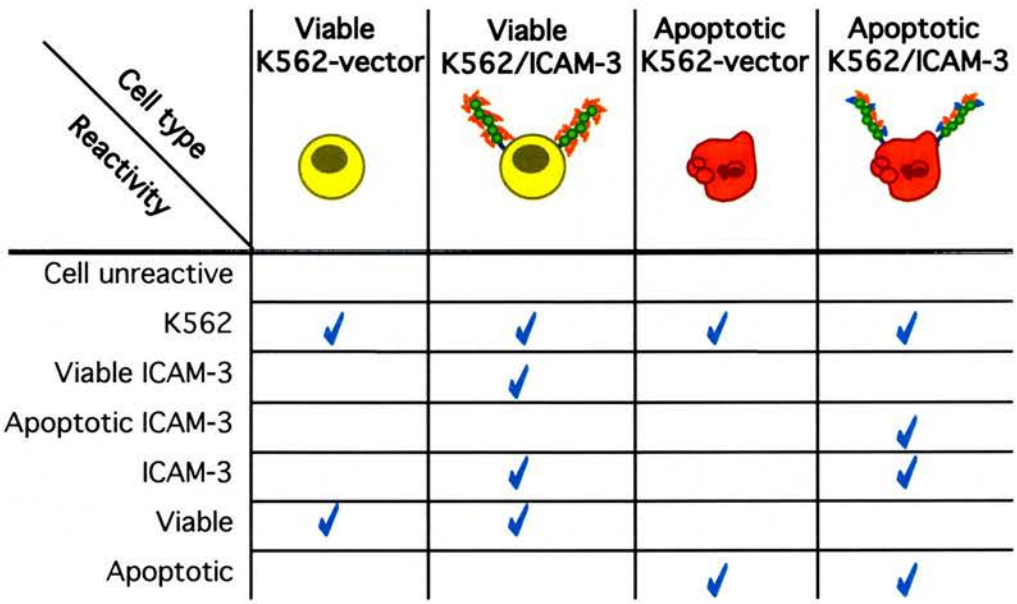


Figure 3-5. Schematic representation of predicted patterns of staining for different theoretical phage clones arising from selection on apoptosis induced ICAM-3 expressing K562 cells.

K562 cells either transfected with ICAM-3 (K562/ICAM-3) or with empty expression vector (K562-vector) were either induced into apoptosis (apoptotic) or left untreated (viable). The reactivity of individual clones from the third round of selection on apoptosis-induced K562/ICAM-3 cells was characterised by analysing the ability to bind each cell type by flow cytometry.

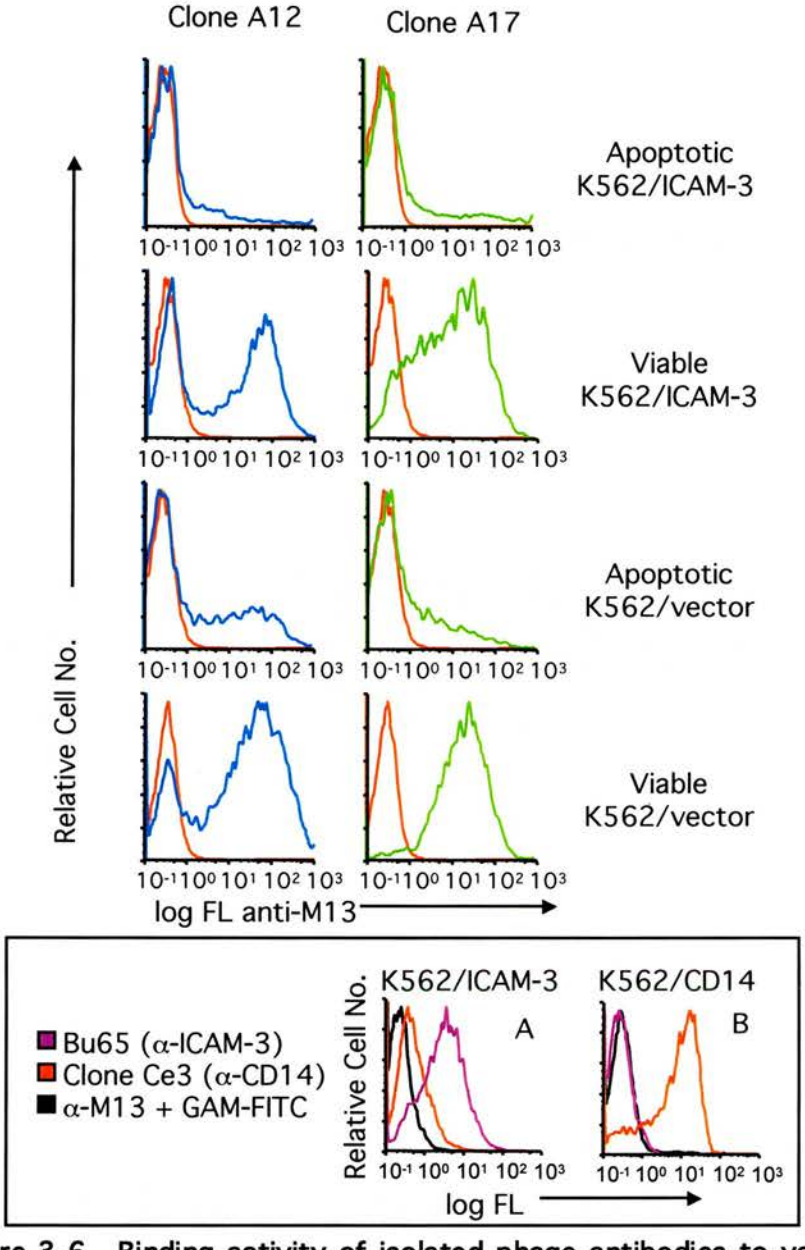


Figure 3-6. Binding activity of isolated phage antibodies to various cell lines. Individual clones from the third round of selection on apoptosis-induced K562/ICAM-3 cells were analysed for the ability to bind various cell lines by flow cytometry. Clone A12 is an example of a phage which bound preferentially to viable cell populations (blue histograms). Clone A17 is an example of a phage showing a bimodal pattern of binding to cell populations(green histograms). **A** Expression of ICAM-3 on viable K562/ICAM-3 cells as determined by Bu65 (α-ICAM-3 mAb) binding. **B** The phage antibody clone Ce3 binds to CD14 and was used as a negative control for background staining by phage antibodies during screening (red histograms). Black histograms represent control staining with mouse anti-M13 and goat anti-mouse-FITC antibodies.

cells), an alternative apoptotic-cell associated epitope or common cell epitope (figure 3-5). Figure 3-6 shows an example of two cell-binding clones subjected to such analysis. Approximately 90 cell-binding clones were identified from over 200 tested. All of these were capable of binding viable ICAM-3-negative cells so were not of interest for further analysis. What is more, for all cell-binding phage, a much lower signal was seen on apoptosis-induced populations. This suggests that the antigens selected for were present at lower levels on cells as a result of programmed cell death.

Two common patterns of binding by flow cytometry were observed – clones which gave a bimodal peak and clones which appeared to stain the entire population of viable cells (Figure 3-6, top half).

It was of interest to find out whether the selected sub-library contained a wide range of clones or if just a few clones had been repeatedly chosen and amplified during the multiple rounds. To make an assessment of clone diversity, DNA-fingerprint analysis of the ScFv regions of 45 cell-binding phage was performed. Twenty-five different restriction patterns were observed (see figure 3-7 for examples). Three common restriction patterns were seen, 20 (44%) of which belonged to one group of phage, 3 (7%) to a second and 2 (4%) to a third. Thus clonal variety amongst the selected sub-library, albeit limited, was confirmed.

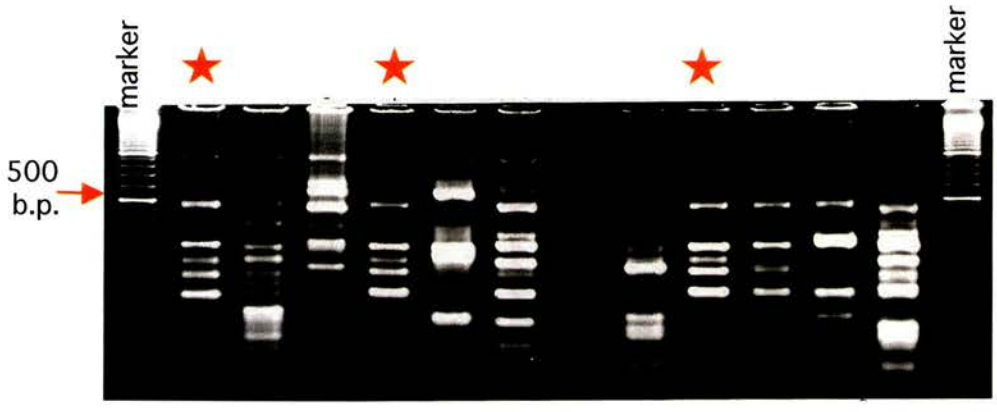


Figure 3-7. Diversity of individual cell-binding phage antibodies from the third round of selection on K562 cells by DNA fingerprint analysis of the scFv genes. The scFv DNA insert of isolated binders was amplified by PCR using plasmid preparations from individual clones as a template. The PCR products were digested with the frequently-cutting restriction enzyme *Bst*N1 and the resulting fragments separated on a 3% agarose gel. A diverse banding pattern was observed, with each unique pattern representing a unique antibody sequence. Stars indicate genetically identical clones. First and last lane is a 100-bp DNA size marker.

3.1.5 Selection of apoptotic-cell-binding phage using populations of mixed viability.

The results from the previous section suggested a requirement for the removal of phage that bind to common cell markers in order to allow for efficient selection of apoptotic-cell or ICAM-3-specific targets. Because of the difficulty in obtaining a pure population of viable cells to use for a standard negative selection one may anticipate the loss of some apoptosis-specific-binding phage on dead cells contaminating the viable cell culture. This section describes a competitive selection approach employed to increase the signal-to-noise ratio for target apoptotic cells, i.e. to increase the number of phage binding specifically to apoptosis-associated epitopes on the cell surface relative to phage binding in an unwanted fashion.

To achieve this, a minor sub-population of "apoptotic" cells was separated from an excess of viable cells in a phage selection mixture. Initial attempts to this effect were made using an excess of viable K562/ICAM-3 cells spiked with a small number of apoptotic K562/ICAM-3 cells. The whole sample was stained with biotinylated Annexin V and the apoptotic subpopulation retrieved using streptavidin-coated beads. However, examination of the cells recovered revealed a substantial number of "trapped" viable cells (results not shown). Instead of optimising the bead-based approach, the decision was made to apply a flow-cytometry based cell-sorting method in order to achieve the same result. This approach has been employed for selections using peripheral blood leucocytes to isolate phage antibodies specific for subsets of B-lineage cells. (Dekruif, Terstappen et al. 1995)

3.1.5.1 The use of Mutu I Burkitt's lymphoma cell line for selection.

Recent studies have failed to show a contribution by ICAM-3 in the clearance of apoptotic K562 cells when the molecule is over-expressed (Shingler 2003). There are many possible explanations for this finding, such as an inadequacy in processing of the molecule by this cell line (which does not usually express ICAM-3) as it dies to bring about the necessary changes for the recognition in its "apoptotic form". Alternatively, the presence of excess ICAM-3, as a result of the over-expression system, could have the effect of masking important epitopes. Nevertheless, populations of Mutu I cells selected for ICAM-3-negativity show decreased interaction with macrophages compared to their wild-type counterparts (Shingler 2003).

For these reasons, Mutu I were chosen for subsequent cell-based phage selections as their engulfment by macrophages in an ICAM-3-dependent manner whilst undergoing apoptosis is well established. In this case, one can be assured of the presence of ICAM-3 in the desired form for the selection.

The approach taken was to sort a minor sub-population of apoptosis-induced ICAM-3 positive Mutu I cells from an excess of non-induced Mutu I cells in a phage selection mixture by flow cytometry.

3.1.5.2 Results

A number of theoretical and practical issues were considered during the experimental design of cell-sorting selections. For example, when deciding the desired overall ratio of apoptotic target cells to viable cells it was important to consider the

contribution of dead cells due to spontaneous apoptosis (which can range from 5-20% in culture of Mutu I cells) amongst the culture used to provide the "viable cell sink". Potential criteria for sorting the dead ICAM-3-positive "target cells" from viable ICAM-3-negative "absorber cells" by flow cytometry considered were: (1) discrimination by light-scatter properties, (2) Annexin V positivity, and (3) ICAM-3 positivity. Initial attempts whereby staining for Annexin V was performed subsequent to incubation with the phage library (carried out on an orbital rotator) led to a significant loss of membrane integrity and cellular fragmentation during the succeeding sort and therefore an inefficient sample collection. Thus, in order to reduce the degradation of cells during the phage selection and cell-sorting, mixing of the selection solution was minimised (by using only gentle inversion of the selection vessel) and apoptotic cells were discriminated on the basis of the light scatter properties. A further modification to previous cell-based selections was the use of 4% w/v BSA in PBS in preference to 4% non-fat dried milk in PBS as a blocking solution after consideration of the potential for structural similarity between ICAM-3 and the numerous glycoprotein structures in milk that could block the specific binding of phage to the target.

The profile obtained from titrating the number of c.f.u.s returning from four rounds of selection on apoptotic Mutu I cells by flow cytometry (figure 3-8) did not mirror the trend observed previously for cell-based selections (figures 3-2 and 3-3). Furthermore, no signal was observed when either polyclonal or monoclonal preparations of phage were tested for their ability to bind either viable or apoptotic cells (data not shown), suggesting that cell-specific phage were not being enriched using this approach.

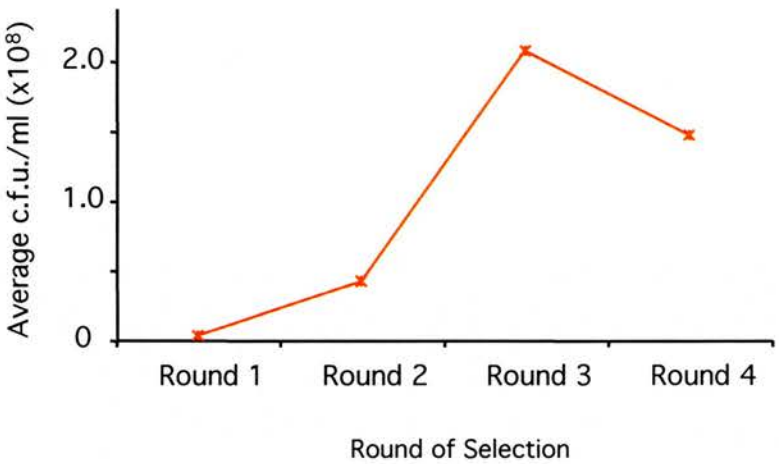


Figure 3-8. Results of selection of phage antibodies by flow cytometric cell sorting of apoptosis-induced Mutu I cells. Phage were titred by infection of eluted particles from each round of selection into *Escherichia coli* TG1 and plating serial dilutions onto ampicillin-containing agar plates. The profile for sequential rounds of selections by flow cytometry did not mirror the profile observed previously for cell-based selections (figures 3-2 and 3-3).

Upon reflection, it was suspected that the choice of whole apoptotic Mutu I cells as a selection target was destined to be problematic as this cell-line became very fragile during later stages of death. It is possible that loss of membrane integrity consequent to the physical stress of a selection process allowed phage to enter and be retained within cells, hampering enrichment of the desired clones. Considering this, and in light of the results of selections on K562 cells, the decision was made to discontinue whole-cell-based selections and place focus on selections employing purified preparations of apoptotic-cell-associated molecules, results of which are presented in the following chapter.

The findings of this chapter can be summarised as follows:-

- Phage antibody selections on K562/ICAM-3 cells demonstrated the successful enrichment of antibody fragments with high affinity for cell-surface epitopes.
- Characterisation of the specificity of cell-binding phage from these revealed a selection of common cell-binders had occurred.
- Furthermore, of the epitopes selected for, all proved to be present at higher levels on viable cells than apoptotic cells.
- Targeting selection to apoptosis-specific epitopes using cell populations of mixed viability proved difficult.

4 Results Chapter 2 - Selection Of Phage Antibodies Using Purified Target Antigens and Cell-Derived Liposomes

4.1 Selections of Phage Antibodies Using Purified Recombinant ICAM-3

4.1.1 Introduction

Data presented in the previous chapter demonstrates that the phage library used for these studies can be manipulated in a way suitable for the isolation of a diverse range of antibody fragments capable of binding to components similar to those utilised for selecting them. However it was also apparent from the screening performed that very few, if any, clones amongst the selected populations had the desired property of being specific to ICAM-3 despite using cells over-expressing the molecule during the selection stage.

This section documents an attempt to increase the concentration of ICAM-3 present during the panning steps by using purified recombinant forms of the molecule as a selection tool. It was proposed that by separating ICAM-3 from other cellular antigens in this way, phage antibodies specific to the molecule would rapidly be recovered. Indeed a phage antibody specific to ICAM-1 was obtained from this library following three rounds of selection on solid-phase bound ICAM-1-Fc fusion protein (Dekruif, Boel et al. 1995; Pierce 2000).

4.1.2 Objectives

As part of a step-wise approach, the initial aim was to demonstrate selection of phage antibodies to purified ICAM-3 derived from viable cells which could also lead to the identification of ICAM-3 antibody capable of blocking apoptotic-cell recognition by macrophages in a similar way to those used initially by Moffatt *et al.* before transferring the system to selections on ICAM-3 purified from apoptotic cells.

4.1.3 Results

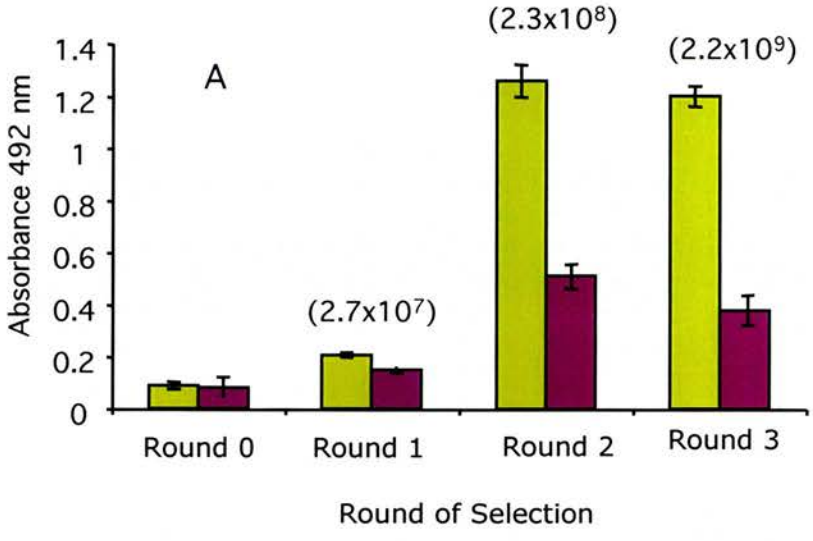
The ICAM-3 fusion proteins used in the studies described below were constructed from the two most membrane distal Ig-like domains of the molecule. These domains were fused to either a human IgG1 Fc fragment (ICAM-3-Fc) or to a peptide containing a polyhistidine and V5 tag (see appendix 4-1).

4.1.3.1 Selection of phage antibodies using ICAM-3-Fc passively adsorbed to immunotubes.

Initial selections were performed using recombinant ICAM-3-Fc fusion protein coated directly to immunotubes (a gamma-irradiated polystyrene tube of similar composition to standard microtitre plates used for protein ELISAs (see materials and methods section 2.12.3.2). Following three rounds, polyclonal preparations of phage from each selection were analysed for specific binding to ICAM-3 in an ELISA. An increase in signal was seen for a polyclonal preparation of phage from the second round of panning compared to the first round, with a comparable ~5-fold increase in the number of clones returning (Figure 4-1 panel A). A further ~10-fold increase in phage number following the third round without an increase in signal for binding to

ICAM-3-Fc by ELISA, suggested that duplication of clones was occurring without further enrichment of target binders (Figure 4-1 panel A). However, subsequent screening and analysis of individual clones revealed that phages bound equally well to the control fusion protein, CD14-Fc (Figure 4-1 panel B). Thus, it appeared that the selection of phages had been directed towards the non-desired Fc portion of the fusion molecule. The trend of increased binding of polyclonal phage preparations to mouse IgG1 as well as ICAM-3-Fc (albeit lower amounts) that was observed with sequential rounds of selection (Figure 4-1 panel A) may also reflect Fc reactivity. One explanation for this result is an unfavourable orientation of the fusion protein during adsorption to the plastic. It was thought that this might have led to the Fc region becoming the dominant epitope during selection. With this in mind an attempt was made to orientate the fusion protein such that the desired portion appeared most distal to the support by capturing the Fc region using protein A coated beads.

After three rounds of selection in this format no characteristic increase in numbers of phage returning from selection was observed and polyclonal analysis by ELISA failed to detect enrichment of target antigen binders (data not shown). A more detailed search of the literature revealed the little-known fact that protein A can bind to an epitope exposed on the heavy chain variable region of single chain antibody fragments (Vidal and Conde 1985; Akerstrom, Nilson et al. 1994). Thus, during the selection protein-A would have been binding to phage antibodies rather than phage just binding to the captured ICAM-3 Fc.



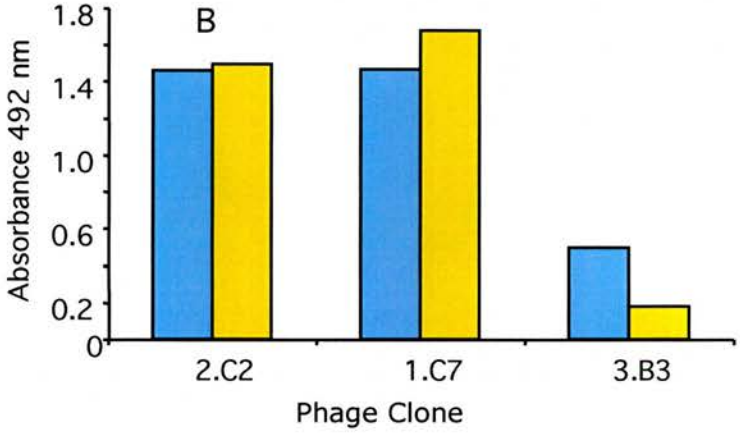


Figure 4-1 Analysis of phage antibodies returning from selections using ICAM-3-Fc adsorbed to immunotubes as determined by ELISA.
A Polyclonal preparations of phage prepared from the clones returning from each round of selection were analysed for binding to ICAM-3-Fc (green) and mouse IgG1 (purple) as a control. After the second round of selection, preferential binding to ICAM-3-Fc was observed. Results shown are the mean +/- S.D. of triplicate wells. Values in brackets above bars for rounds 1-3 represent the number of phage (c.f.u./ml) returning from each selection. **B** Individual phage clones from round three of selection for binding to either ICAM-3-Fc (blue) or CD14-Fc (yellow).

4.1.3.2 Selection of phage antibodies using polyhistidine-tagged-ICAM-3 captured on nickel-charged magnetic beads.

Bearing in mind the apparent tendency to select for Fc-binding phage, an alternative ICAM-3 fusion protein that contains a polyhistidine tag (referred to from hereon as His-I3) was employed as the selection agent. It was hoped that this construct would increase the efficacy of obtaining binders to the target in several ways. Firstly, it provides an increase in the proportion of target within the fusion protein as the peptide tag only adds 3kDa to the target as compared to approximately 25kDa provided by the Fc partner.

In addition the polyhistidine moiety provides advantages relevant to selection protocols. At the immobilisation stage it would allow correct orientation to a nickel-charged solid phase. Furthermore, following selection it would allow removal of the protein with bound phage from the support by competitive elution from the nickel using imidazole, leaving behind phage bound to the support (figure 4-2).

4.1.3.2.1 Optimising the coating of His-I3 to nickel-charged beads

Following the production and purification of His-I3, three rounds of selection were performed using beads coated with the fusion protein. Criteria such as binding capacity and incubation conditions recommended by the manufacturers were followed to coat beads. However, this resulted in an enrichment of phage that bound to the surface of nickel-charged beads (figure 4-3) but failed to bind to ICAM-3-coated microtitre plates by ELISA (data not shown). This suggested that the beads had not been optimally coated prior to selection allowing phage to bind to the nickel-

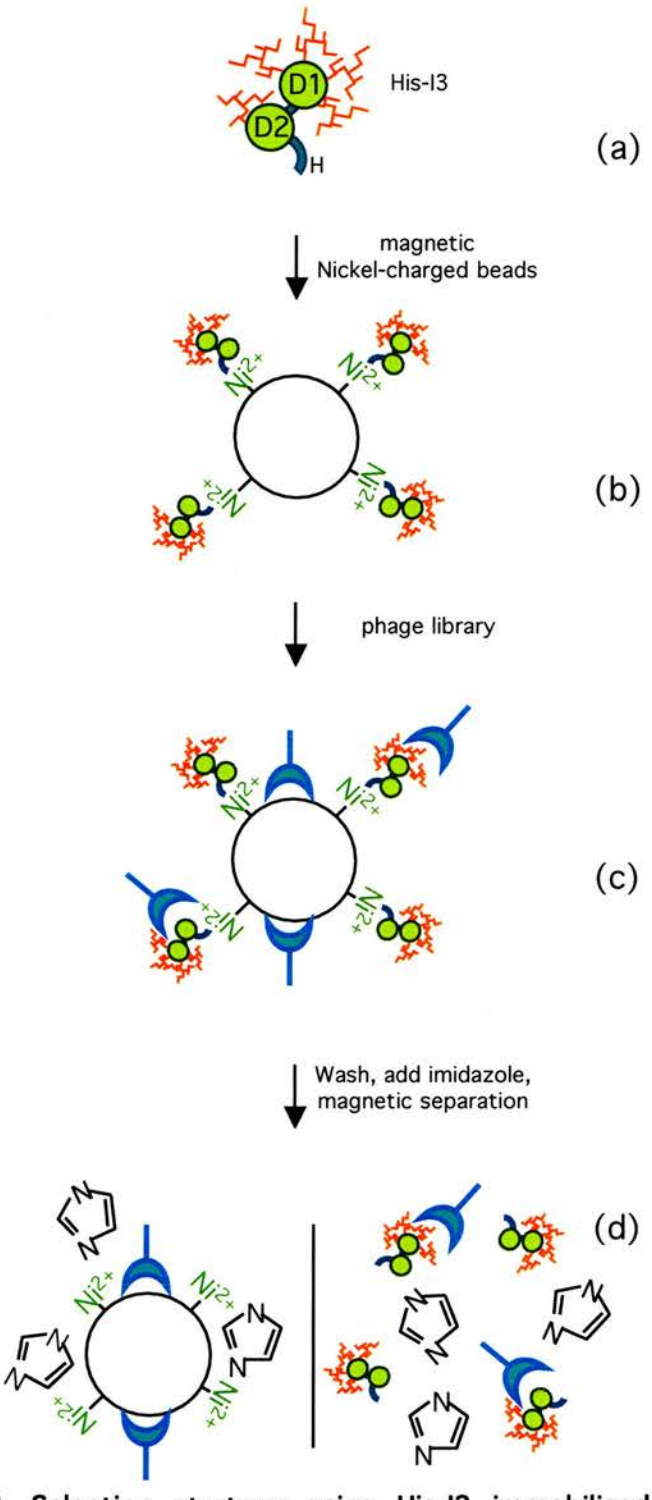


Figure 4-2. Selection strategy using His-I3 immobilised on nickel^{2+}-charged paramagnetic beads. (a) Domain 1+2 of ICAM-3 fused to a polyhistidine (H) tag (His-I3) is captured on Ni^{2+} charged paramagnetic beads. (b) His-I3 coated beads are incubated with the phage library allowing phage to bind to both His-I3 and the bead surface. (c) Following wash steps to remove unbound phage, His-I3 with attached phage is selectively eluted from the bead surface using imidazole. Beads are separated from the eluted material by virtue of their paramagnetism.

charged sites on the surface. Thus it was necessary to optimise bead coating. Figure 4-4 panel B shows an example of a bead-based ELISA using binding of an antibody to ICAM-3 (Bu65) as the readout for monitoring the coating of individual batches of fusion protein.

As the fusion protein with phage bound was going to be eluted following selections on the basis of its nickel binding, it was necessary to confirm that the beads were coated exclusively in this way and that the His-I3 had not passively adsorbed to the surface. To demonstrate this an ELISA was carried out on beads coated with the fusion protein and then subjected to elution with imidazole or left untreated. Figure 4-4 panel D shows that no ICAM-3 could be detected on the beads following incubation with the fusion protein preparation at concentrations required for use within a selection.

Prior to use in phage selection, suitable conditions for bead coating were established for each batch of recombinant protein. As relatively high protein concentrations were required for bead coating, the number of beads used for selections was kept to a minimum to ensure a single batch of fusion protein could be used for all of the necessary rounds of selection and screening.

Following one round of selection relatively few phage (~3200 clones) were recovered. This may have been due to the small quantity of beads and thus antigen present during the selection. With the knowledge that enrichment can be seen following a single round of selection if highly purified antigen has been used (personal communication, Jane Wilton, Cambridge antibody technology), a decision was made not to continue with further rounds of selection but instead proceed to screening monoclonal phage for their ability to bind ICAM-3-Fc by ELISA.

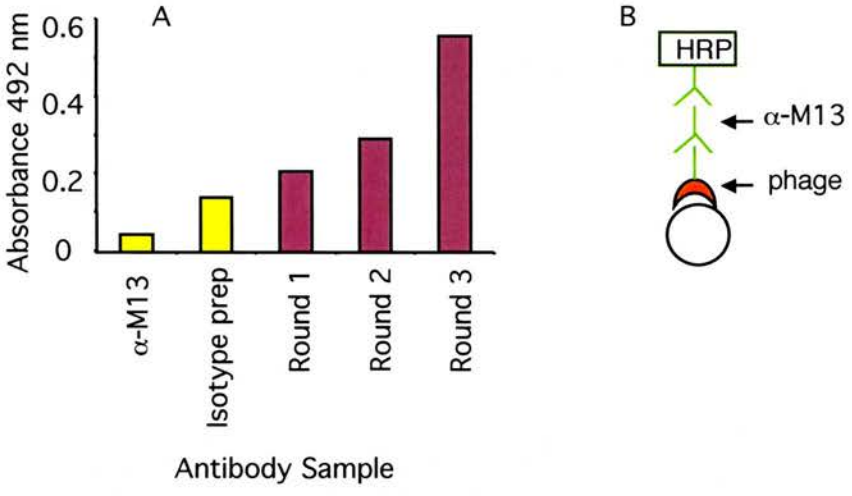


Figure 4-3. Suboptimal coating of beads leads to selection of phage capable of binding to free nickel-charged sites on the bead surface
A Polyclonal preparations of phage prepared from the clones returning from each round of selection (purple bars) were analysed for binding to uncoated nickel-charged magnetic beads. The binding of polyclonal preparation of phage from a cell-based selection (isotype prep) and anti-M13 antibody was assessed to determine the level of background binding (yellow bars). Data in A is representative of two independent assays. **B** Schematic representation of the bead-based ELISA. HRP; horseradish peroxidase.

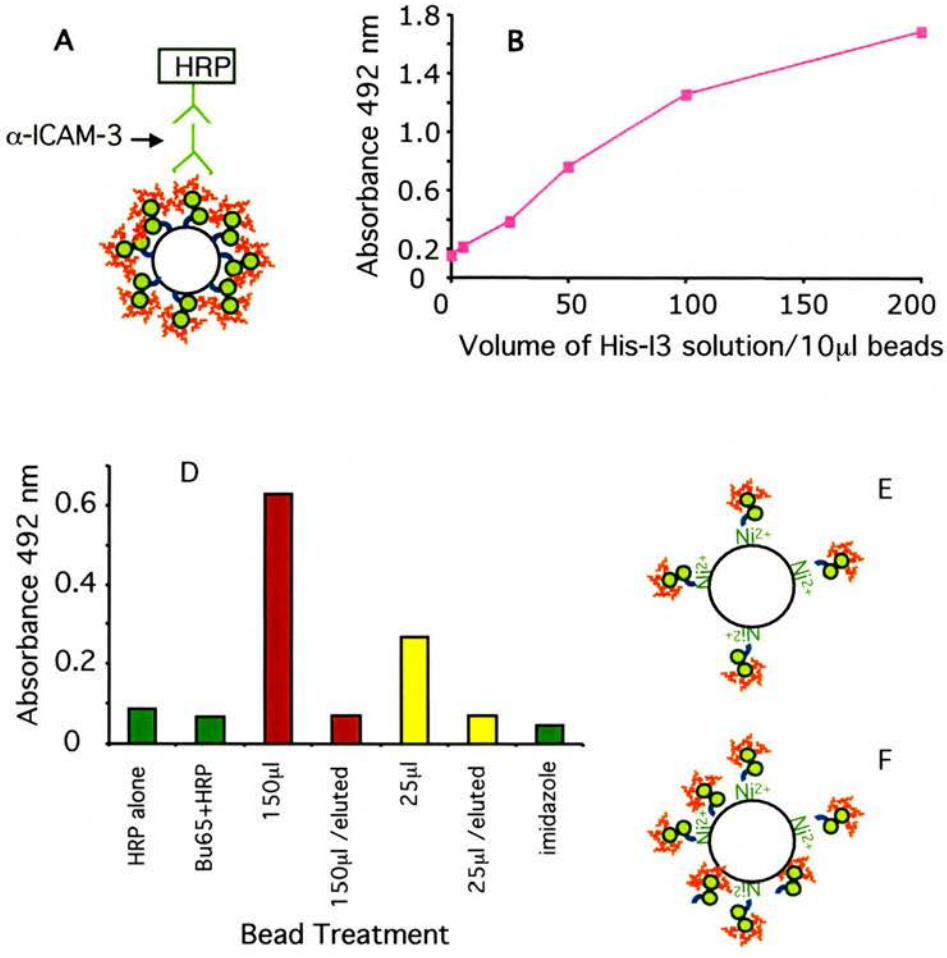


Figure 4-4. Optimisation of bead coating with His-I3 as assessed by binding of antibody to ICAM-3 in a bead ELISA.
A Schematic representation of the bead-based ELISA used to assess the level of ICAM-3 associated with beads. Following bead-coating with His-I3, mouse monoclonal antibody BU65 (α-ICAM-3) was used to detect ICAM-3. HRP; horseradish peroxidase. **B** Various volumes of a purified preparation of His-I3 were to coat a fixed volume of nickel-charged beads before assessing bead-associated ICAM-3 by ELISA. **D** To determine whether all detectable His-I3 was bound to the bead surface specifically via the nickel-charged sites (as in **E**) as opposed to being passively adsorbed (as in **F**), beads were coated with various volumes of purified His-I3 (150μl or 25μl), then either treated with imidazole or left untreated before making an assessment of bead-associated ICAM-3. Data in B and D are representative of three and two independent assays respectively.

Considering that ~1500 copies of each clone in the library were present during the selection it was reasoned that screening of ~600 of the individual clones should provide a representative indication of the total population of clones selected.

Approximately 75 clones from 600 tested bound to ICAM-3-Fc by ELISA. However, subsequent analysis revealed that they also were able to bind to preparations of CD14-Fc. Considering both His-I3 and I3-Fc were produced using the same expression system (transiently transfected HEK 293^T cells) and that both preparations contained significant levels of other proteins, as determined by viewing of Coomassie blue-stained SDS-polyacrylamide gels (most notably a band at 20-25 kDa of unknown identity. See also section 4.1.3.2.2 and appendix 4-2), it is conceivable that phage were being selected through a common contaminant present in both the Fc- and His-tagged fusion protein preparations.

4.1.3.2.2 Optimising antigen purity

Initially the amount of His-I3 purified preparations had been estimated by measuring total protein concentration in a Bradford dye-based assay. However, subsequent analysis revealed His-I3 to be only a minor component of the samples obtained from the purification step (< 1% of total protein in a standardised ICAM-3 ELISA). It was suspected that these contaminating components were also chelating the nickel on the column used to purify the fusion protein. The same may also have been true for ICAM-3-Fc purification. An alternative explanation is that soluble proteins that associate directly with ICAM-3 could have been co-purified with the fusion protein. These could have originated either from serum in the culture medium or have been secreted from cells during growth.

To circumvent the problem of contaminating serum proteins providing a selection impediment, fusion proteins were produced in serum-free medium. A number of commercially available products were assessed for maximum fusion protein productivity, and one was chosen, which only contained recombinant human insulin as a source of protein.

Following the purification of proteins produced under these conditions, protein bands in addition to those of ICAM-3 (and not the size expected for recombinant insulin) were still observed (appendix 4-2), suggesting contamination by cell-derived molecules in the conditioned medium.

4.1.3.2.3 Screening and characterisation of clones returning from selections on His-I3 produced in serum free conditions.

Given that the technical requirements for further purification were beyond those readily available, the decision was made to continue with the selection of phage using the His-I3 preparation derived from serum free cell culture despite the persistent presence of contaminating proteins.

Following one round of selection ~600 clones were screened (approximately 15% of the total number returning from the selection). Nearly 80 of these clones showed a clear signal in an ELISA directed against ICAM-3-Fc. However, subsequent analysis revealed that these could also bind CD14-Fc. The phage did not show a high affinity towards uncoated ELISA wells demonstrating that they were not merely binding to plastic.

As no Fc portion should have been present during the selection, phage must have been selected through epitopes present in all of the fusion protein preparations.

One explanation is that phage were being selected through regions in His-I3 conserved amongst other Ig domains allowing them to cross-react with similar epitopes found on the Ig-domain-containing proteins in the screening assays.

Such specificity may be of diagnostic/therapeutic use, perhaps in discriminating Ig-domain expressing cells from non-Ig domain expressors or for masking all Ig domains. To address this hypothesis, a panel of phages giving the highest signal in the initial ELSIA screen were tested for their reactivity to a panel of Ig and non-Ig domain-containing antigens.

A weak preference towards Ig domain-containing antigens as compared to antigens not containing an Ig-domain was seen for several clones (figure 4-5). However, the background signal observed for non-Ig containing antigens was higher than for the control phage clones Ce3 (anti-CD14) (figure 4-6) suggesting the clones have a low affinity for these antigens too.

When tested for the ability to bind to Ig domain-expressing cells, no clones showed reactivity by flow cytometry or microscopy (data not shown).

Explanations for this observation include the possibility that the clones only bind antigen when they are immobilised or that the read-out from the ELISA is more sensitive than immunofluorescence by microscopy or flow cytometry.

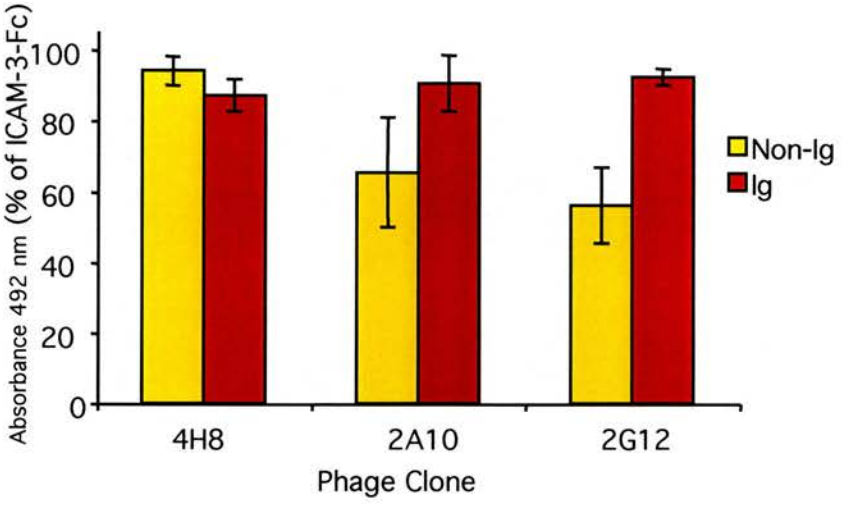


Figure 4-5. Some clones selected using His-I3 preferentially bind to Ig-containing protein preparations in a direct ELISA.
Wells were coated with solutions (10μg/ml) of either Ig-containing antigens (CD14-Fc and mouse IgG) or non-Ig-containing antigens (bactotryptone and LPS). Phage clones were analysed for the ability to bind antigen coated wells in an ELISA. Results represent the combined mean +/- S.D. for each class of antigen (duplicate well for individual antigen) and are shown as a percentage of binding to ICAM-3-Fc.

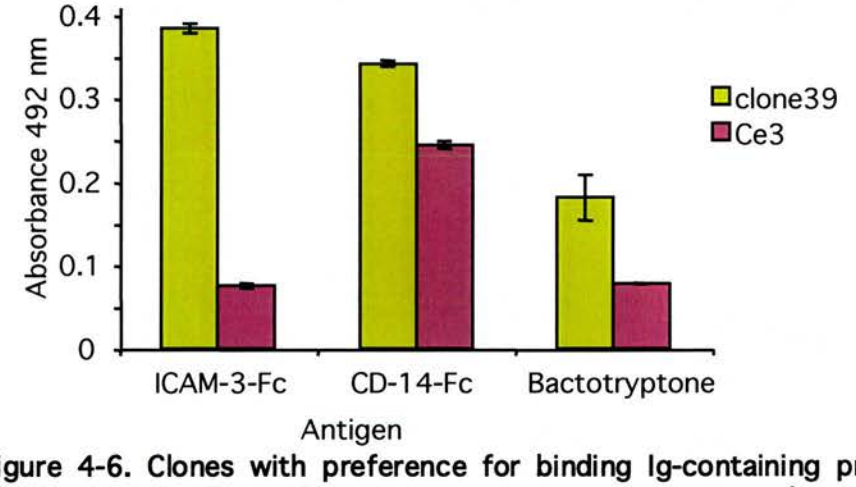


Figure 4-6. Clones with preference for binding Ig-containing proteins show a higher affinity for non-Ig containing preparations than control clones.
Wells were coated with solutions (10μg/ml) of either Ig containing antigens (CD14-Fc or ICAM-3-Fc) or the non Ig containing antigen, bactotryptone. Phage clones were analysed for the ability to bind antigen coated wells in an ELISA. Clone Ce3 is specific for CD14. Results shown are the mean +/- S.D. Of duplicate wells.

The results of this section can be summarised as follows,

- Following the selection of phage antibodies with purified preparations of recombinant ICAM-3, enrichment of clones capable of binding to components of the purified preparation was observed.
- Phage antibodies selected using purified preparations of recombinant ICAM-3 also displayed an equal affinity for similar, non-ICAM-3-containing preparations of proteins.
- The binding properties of clones obtained from selections performed on His-tagged ICAM-3 suggested that antibody fragments were being selected through conserved epitopes on Ig-domains
- Clones binding to immobilised protein preparations did not display cell binding.

Thus, targeting of selections towards domains 1 and 2 of ICAM-3 using purified preparations of this protein in recombinant form was not successful. In order to demonstrate that purified antigens can be used to isolate cell-binding antibody fragments from the phage library used in this work, selections were performed on an alternative apoptotic-cell-specific marker, namely phosphatidylserine. The results of these selections are presented in the following section.

4.2 Selection of Phage Antibodies Specific to Phosphatidylserine

4.2.1 Introduction

Currently, the most definitive means of detecting surface changes associated with apoptosis is the persistent exposure of phosphatidylserine (PS). Despite the large number of receptors and bridging molecules of the innate immune system that have been suggested to bind PS (Savill, Hogg et al. 1992; Hanayama, Tanaka et al. 2002; Anderson, Maylock et al. 2003), until very recently, no good-quality monoclonal antibodies to this phospholipid have been developed (Mandinov, Mandinova et al. 2003).

The binding of Annexin V, the fifth member of a structurally related family of proteins (Reutelingsperger, Kop et al. 1988; Tait, Gibson et al. 1989; Crumpton and Dedman 1990), by apoptotic cells has been employed as a trusty marker of programmed cell death for almost a decade (Vermes, Haanen et al. 1995). However the requirement for calcium to be present at relatively high concentrations for this binding to occur hampers its use in many systems.

An anti-PS antibody or, in particular, antibodies against specific portions of the molecule would allow more detailed studies into the structure–function relationship of the changes to PS, such as alterations in its oxidation state that are known to occur during apoptosis (Kagan, Borisenko et al. 2003). Moreover, identification of a neutralizing antibody specific to "apoptotic PS" would allow *in vivo* discrimination of effete cells from viable cells that may have external PS as a result of other

physiological phenomena, such as during the activation of B cells (Dillon, Mancini et al. 2000).

The presence of naturally-occurring PS-autoantibodies has been linked to many autoimmune disease states, notably systemic lupus erythematosus (SLE) (Toschi, Motta et al. 1993). In such situations "antiphospholipid syndrome" can lead to a number of disorders such as end stage renal disease (Vaidya, Daller et al. 2002). These antibodies often display heterogeneity in binding specificities, varying in their dependency on oxidation states and the presence of protein cofactors. The antigenic epitopes for the autoantibodies appear mainly to be the polar head groups of the phospholipids suggesting a structural basis by which they can cross-react with DNA (Cocca, Seal et al. 2001).

Under normal circumstances, lipids are considered to be very poor immunogens when administered by conventional means (Boullerne, Petry et al. 1996), presumably due to the requirements for antigen processing during an immune response. Phage antibodies provide an alternative approach for isolating such specificities. Recently, a phage-displayed semi-synthetic human antibody library was used successfully to isolate antibodies specific to methyl palmitate, a major component of bacterial glycolipids and lipoproteins in animal cells. The selected single-chain variable fragments were able to prevent micelle formation of lipoteichoic acid from Gram-positive bacteria, inhibit lipopolysaccharide-induced tumor necrosis factor alpha release in mononuclear cells and bind to hydrophobic bacterial surfaces (Gargir, Ofek et al. 2002). These precedent results illustrate the potential of using phage to produce novel anti-PS antibodies. The work detailed in this section therefore aimed

to apply phage antibody technology in a similar way to obtain anti-PS antibody fragments with potential diagnostic or therapeutic applications relevant to apoptosis.

To this end, a suitable means for immobilising PS was sought prior to using solid-phase-bound PS as a selection agent. PS-binding phage were screened for their ability to bind apoptotic cells.

4.2.2 Results

Functional studies involving purified phosphatidylserine generally involve the production of liposomes. As it is accepted that liposomes made purely from PS are unstable in solution, PS usually represents a minor component of these small lipid vesicles (~30%) compared to other phospholipids (typically phosphatidylserine PC) which act as PS carriers (Fadok, Voelker et al. 1992). Preliminary selection of PS-specific liposomes was carried out using PS/PC liposomes in solution. Due to the low sedimentation coefficient of these small particles long high-speed centrifugation was required to pellet the liposomes during the washing stages. Minimal success was achieved in obtaining phospholipid-specific phage, and of the few identified, none were specific to PS (data not shown). It was reasoned that PC was dominating the selections. The work described below attempted to circumvent these obstacles by performing selections using immobilised PS alone.

4.2.2.1 Immobilisation of PS directly to gamma-irradiated polystyrene and subsequent binding of Annexin V

Immobilisation of lipids for ELISAs usually involves either the addition of the lipid in ethanol followed by air-drying or their coupling to protein molecules.

The solvent used for immobilisation can affect the orientation and spatial packing of lipids to plastic and covalent coupling to protein decreases the relative amount of the target-lipid to support. As the end use of immobilised PS in these studies was to select for phage capable of binding apoptotic cells the capacity of phospholipids to coat plastic in an aqueous phase (in the absence of organic solvent) was tested.

The ELISA results shown in figure 4-7 reveal that PS can bind directly to gamma-irradiated polystyrene microtitre plates for the specific detection by Annexin V. A simple short incubation of the lipid dissolved in an aqueous serum-free solution (Hank's Balanced Salt Solution, HBSS) as a buffer at 37°C, resulted in a stable coating that was suited for use in ELISA. Furthermore, Annexin V binding capacity was maintained following 20 washing steps with PBS as an imitation of the rigorous procedure required to remove non-specifically bound phage following a selection. BSA in HBSS was the chosen blocking solution for both the unoccupied sites on the solid support and for the phage library in subsequent selections. This was used in preference to the popular blocking agent, dried milk powder, as although being marketed as "non-fat", phospholipids could still be present in low amounts.

4.2.2.2 Enrichment of phospholipid-specific phage

Following the ELISA result confirming firm immobilisation of phospholipids, selection of phage particles displaying phospholipids-reactive antibody fragments was conducted by panning against PS-coated immunotubes. Immunotubes were coated with PS and blocked using the concentrations and conditions used to establish the plastic-binding capacity (see also section 2.12.3.2).

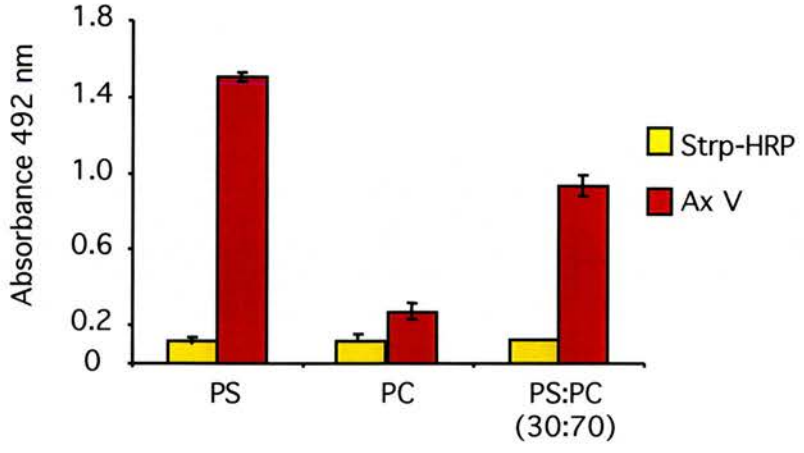


Figure 4-7. Preservation of the Annexin V binding epitope of phosphatidylserine in an ELISA format. Phospholipids were resuspended in serum-free HBSS and used to coat gamma-irradiated polystyrene microtiter ELISA plates prior to analysis using biotinylated Annexin V and detection with streptavidin HRP. Wells were coated using solutions of phosphatidylserine (PS), phosphatidylcholine (PC) or a 30:70 mixture of PS:PC. Results shown are the mean +/- S.D. of duplicate wells. Representative of two independent assays.

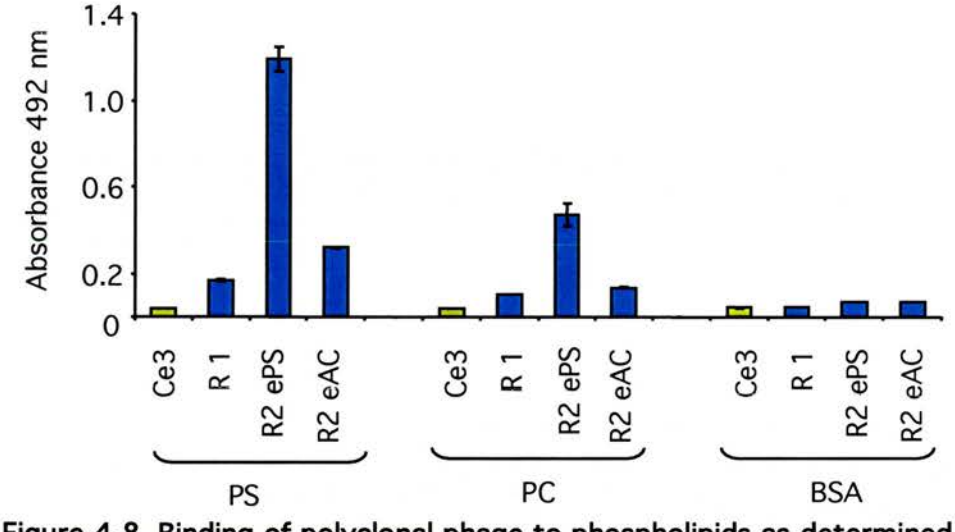


Figure 4-8. Binding of polyclonal phage to phospholipids as determined by ELISA. Bacterial culture supernatants containing phage were prepared from the first and second round (R1 and R2 respectively) of selections on PS-coated immunotubes, and analysed for binding to PS, PC as a control phospholipid or the irrelevant antigen, BSA. After the second round of selection, preferential binding to PS was assessed for phage populations eluted using PS in solution (ePS) or apoptotic cells in suspension (eAC). Ce3 phage antibody supernatant (α-CD14) represents background binding. Results shown are the mean +/- S.D. of duplicate wells.

4.2.2.2.1 Elution of phospholipid-bound phage

A range of elution strategies was employed during the various rounds of panning. Following the first round of selection, bound phage were eluted by direct disruption of all ionic interactions in a low pH (2.2) buffer to maximise retrieval (and neutralised with 1 M Tris, pH 7.4). For subsequent rounds, an attempt was made to elute just those phage bound to phospholipids by competing phage from solid-phase-supported PS with PS at high concentration in solution. A similar strategy has been successfully used to generate anti-idiotypic antibody fragments capable of mimicking conformational epitopes of tumor-associated antigens (Goletz, Christensen et al. 2002).

For these studies the competitive elutants used were: -

(a) A high concentration of PS in solution prepared in an identical way to that used to coat immunotubes (methods section 2.12.3.2). This was followed by incubation with PC-coated immunotubes to select negatively for broad-spectrum phospholipid binders.

(b) A suspension of apoptotic Mutu I cells with PS exposed on the outer-leaflet of the plasma membrane. The rationale for this was to elute just those phages capable of binding phospholipid in the context of the cell membrane. Cell-bound phage were pelleted and washed before infection into TG1s.

Figure 4-8 shows the results of an ELISA used to monitor enrichment of phospholipid-binding phage after the various selections.

Polyclonal preparations of phage showed a considerable increase in binding to PS-coated wells following the second round. In addition, a stronger signal was seen for PS as compared to PC and there was no significant enrichment of phage capable of

binding BSA-coated wells. These results show that selection of phage on immobilised phospholipid followed by a specific elution strategy can afford a rapid enrichment of target-specific binders.

4.2.2.2.2 Binding characteristics of the selected phage clones

4.2.2.2.2.1 Initial screening and analysis by ELISA

Following the various rounds of panning, selected monoclonal phages were screened in an ELISA for PS. A number of clones reactive to microtitre plates coated with PS were isolated. For clones eluted using a solution of purified PS, 70 of 880 screened (8%) were positive, whilst from the clones eluted using whole apoptotic cells, 14 of 400 screened (3.5%) were positive (table 4-1). The relative proportion of binders between elution methods is consistent with the polyclonal phage ELISA analysis.

In order to evaluate whether a significant portion of ELISA-positive phage had scFv specific for PS, a panel of phage clones giving the highest ELISA levels were tested on plates coated with either the irrelevant antigen, BSA or PC as phospholipid control in addition to PS. When tested in an ELISA format, some phage bound only to PS coated wells (e.g. A3) and some bound to both PS and PC (e.g. B9). Others bound to similar levels to all antigens tested (e.g. H11)(figure 4-9). Thus, multiple clones with contrasting binding specificities had been selected.

4.2.2.2.2.2 Binding of anti-phospholipid phage to Mutu I cells

Based on the ELISA data indicating the presence of immobilised-PS-specific phage antibodies amongst the selected population, individual ELISA-positive clones were further analysed for their ability to bind native PS as it exists on apoptotic Mutu I cells. For clones eluted using a solution of purified PS 2 of 880 screened (0.23% of

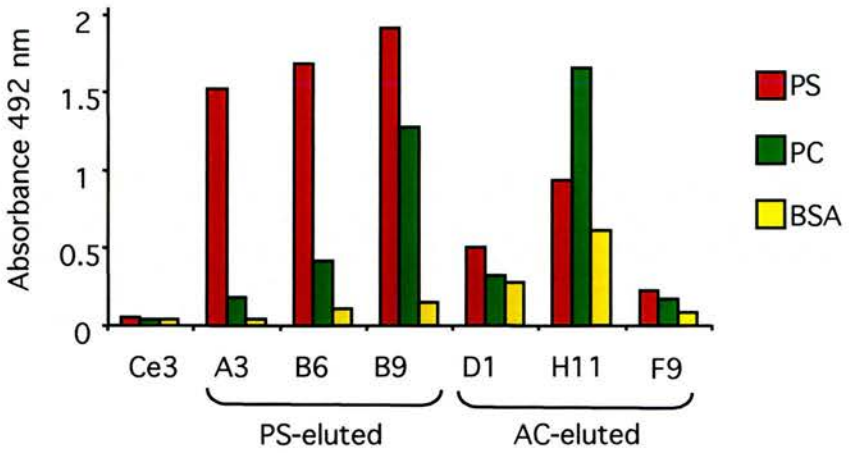


Figure. 4-9. Anti-phospholipid binding characterisation of selected phage clones. Individual scFv clones identified as positive from primary screening were analysed for binding to PS, PC as a phospholipd control or the irrelevant antigen, BSA in an ELISA. Examples of clones from selections where either purified PS in solution (PS-eluted), or apopotic cells exposing PS (AC-eluted) were used during the elution step are shown. Representative of two similar assays.

	PS Solution Eluted			Apoptotic Cell Eluted		
	Total screened	ELISA +ve	Cell+ve	Total screened	ELISA +ve	Cell+ve
No. of clones	880	70	2	400	14	5
% of total screened		8%	0.23%		3.50%	1.25%
% of ELISA +ve			2.86%			36%

Table 4-1. The effect of different elution strategies on the reactivity of PS-selected phage populations towards PS in an ELISA or on apoptotic cells.
Summarised results of screening individual phage clones for the ability to bind purified PS in an ELISA (ELISA +ve) or apoptosis-induced Mutu I cells by flow cytometry (Cell +ve). A higher percentage of phage clones identified as positive for binding PS by ELISA were also positive for binding ACs when eluted from immobilised purified PS using ACs than when eluted using purified PS in solution ($P = 0.0011$, Fischer's exact test).

total, 2.86% of ELISA-+ve) bound the population of Mutu I cells which display a high level of level of AxV binding, whilst from the clones eluted using whole apoptotic cells 5 of 400 screened (1.25% of total, 36% of ELISA-+ve) bound (table 4-1 and figure 4-10). Five clones failed to bind cells following regrowth on a larger scale despite maintaining ELISA reactivity. It is unlikely that these were false positives because original batches showed the same binding pattern when used for staining on separate occasions.

The loss of cell reactivity could have been a result of mutation within variable regions that provided a growth advantage to the bacteria. This is known to occur in non-recombinant deficient strains such as TG1s (personal communication, John Dekruif, see also section 5.5). Perhaps antibody fragments with too strong a specificity for native PS would bind to the phospholipids, which have been shown to be present for several seconds as a synthetic intermediate of PE at cytoplasmic membranes. Considering that this is also the site of phage particle assembly, one could imagine binding to PS here could interfere with normal cellular function. Loss of cell reactivity was not due to decreased sensitivity following a reduction in phage particle production as the total phage content remained the same or even increased after regrowth (see figure 4-11). However, this method only measures total coat protein and is not a measure of phage particles bearing antibody fragments. Alternatively, this could have been due to a reduced level of antibody fragment expression per phage particle resulting in lower avidity effects. Furthermore, failure to regrow specifically-binding clones was not due to outgrowth of another clone contaminating the original culture as individual colonies (streaked onto an agar plate,

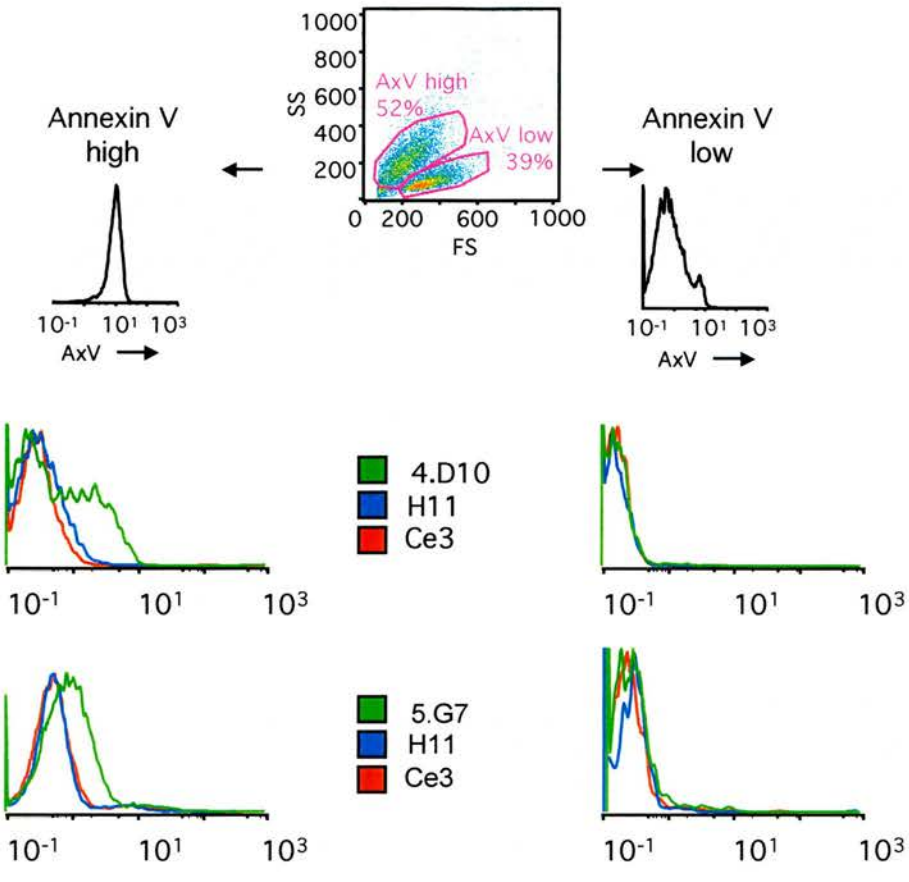


Figure 4-10. Binding of monoclonal phage antibodies to apoptosis induced Mutu I cells. ELISA-positive phage were prepared from individual clones after the second round of selection and analysed for their ability to bind Mutu I cells by flow cytometry. Blue histograms represent staining with clone that produced a strong signal in an ELISA for PS that failed to bind cells. Clones 4.D10 and 5.G7 bound late apoptotic Mutu1cells. Staining with the α-CD14 phage clone, Ce3 is also included as a negative control. Representative of staining performed on two separate occasions with one batch of phage antibody.

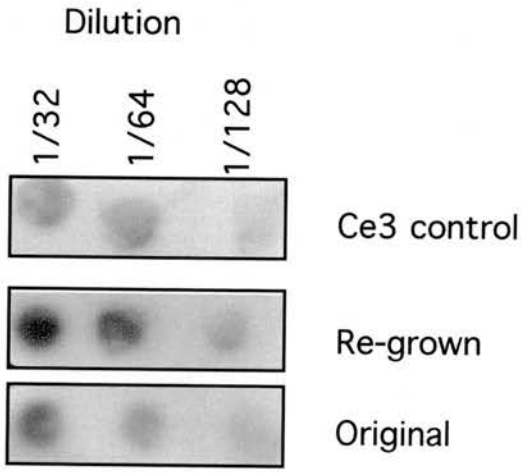


Figure 4-11. Determination of M13 coat-protein concentration by dot-blot analysis. Supernatants of bacterial cultures containing phage particles were serially diluted and aliquots allowed to adsorb to PVDF membrane. The amount of M13-coat-protein present on membranes was visualised using a peroxidase conjugated antibody to the protein. A preparation of the phage clone, Ce3 was included as a positive control to gauge the amount of M13-coat-protein required within a sample in order to see cell binding. Representative of three separate experiments.

grown up and tested for binding) all tested positive by ELISA but were still negative on cells. Nevertheless, clones 12.D12 and 13.F2 maintained cell reactivity upon re-growth, albeit with low signals (figure 4-12). Low-affinity antibodies are a feature of naïve phage libraries, and this problem could have been compounded by the elution strategy employed during these selections. These clones were analysed by ELISA for phospholipid specificity. Although clone 12.D12 showed specificity towards PS, surprisingly, clone 13.F2 bound equally as well to PS as to PC (figure 4-13).

This raises the question of why clone 13.F2 only bound to apoptotic cells and not the PC in the outer leaflet of viable cell plasma membranes. There are several possible explanations for this observation. It could be that due to changes in membrane fluidity, lipid packing or oxidation, the exposed phospholipid epitopes on apoptotic cells are more similar to those exposed on plastic-immobilised phospholipids as detected by ELISA than on "unstressed", viable cells. Alternatively, phage particles could have been entering cells and cross-reacting with other sites bearing similar epitopes to phospholipids such as the phosphate groups of DNA. Or perhaps clone 13.F2 simply doesn't bind to PC in the conformation found on the viable cell surface. Whatever the mechanisms, these studies have demonstrated that cell-binding antibody fragments can be isolated from clones selected using immobilised purified antigen.

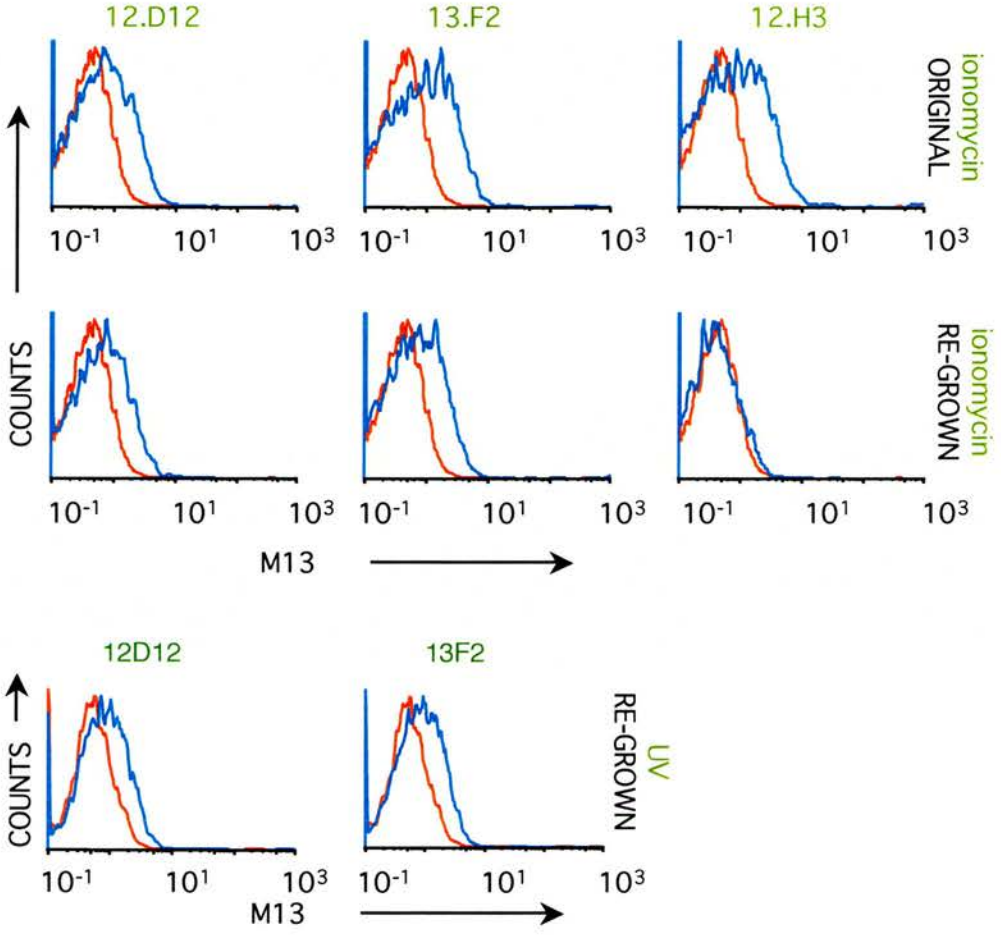


Figure 4-12. Binding of "re-grown" monoclonal phage antibodies to apoptosis-induced Mutu I cells. The binding capacity of original and "re-grown" monoclonal phage preparations was compared by flow cytometry. Clones 12.D12 and 13.F2 reproducibly bound to late apoptotic cells (when produced on three independent occasions). Clone 12.H3 is an example of a clone which displayed loss of cell binding capacity following re-growth. Clones 12.D12 and 13.F2 bound equally well to ionomycin and UV induced Mutu I cells. Staining with the α-CD14 phage clone, Ce3 is also included as a negative control (Red histograms). Representative of cell-staining performed using phage prepared on three independent occasions. Representative of staining performed on two separate occasions for each batch of phage antibody.

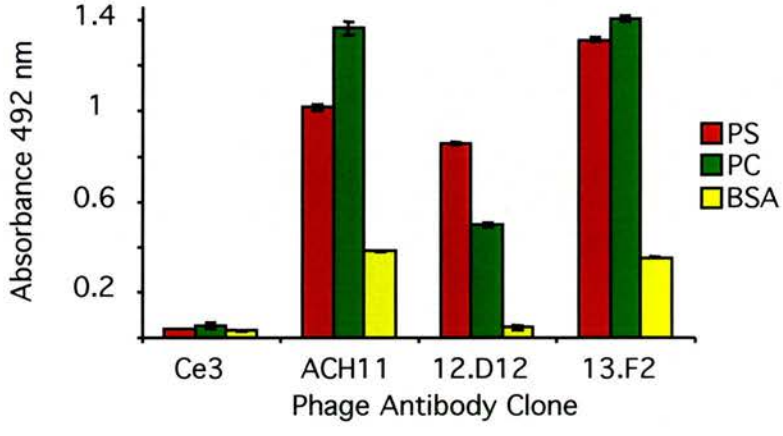


Figure 4-13. Anti-phospholipid binding characterisation of cell-binding phage clones. Cell-reactive scFv clones were analysed for binding to PS, PC as a phospholipid control or the irrelevant antigen, BSA in an ELISA. ACH11 represents a clone that showed phospholipid binding capability in an ELISA but not to cell membranes. Results shown are the mean +/- S.D. of duplicate wells. Representative of two similar experiments.

4.2.3 Selection on lipid symmetric erythrocytes

Although it is possible that further screening of clones from the sub-library collected after two rounds of selection on immobilised PS could have led to the identification of a high affinity PS-specific cell-binding antibody fragment, it was decided to use an alternative source of membrane-bound PS for further selections. Erythrocytes from normal human peripheral blood lose their membrane asymmetry following osmotic treatment in the presence of calcium. This results in exposure of PS on the outer leaflet of the cells without the gross changes in the cytoskeletal network associated with apoptosis (Schlegel, McEvoy et al. 1985). In addition, these cells lack a nucleus and are much smaller than lymphocytes, so have a higher ratio of cell-surface membrane to cytoplasm. For these reasons they were chosen as a tool for further selection of PS-specific antibodies.

Figure 4-14 shows analysis of the PS-binding capacity of polyclonal phage preparations from a round of selection on lipid-symmetric erythrocytes using sub-libraries derived from the earlier phage selections on immobilised PS. No increase in binding to purified PS by ELISA was observed. The slight decrease could be explained by the loss of phages that preferentially bind to PS, as it exists immobilised on plastic, whilst enriching for cell-binding phage.

No reactivity of polyclonal preparations to apoptotic cells was observed, (data not shown). However, experiments demonstrated that up to 1 in 16 cell-binding phage are required in a standard phage preparation to see a signal by flow cytometry (data not shown). Therefore, in the event that the number of cell binding phages within the polyclonal population was just below detection limits, individual cones were screened for cell binding. However, no enrichment of high affinity cell-binding

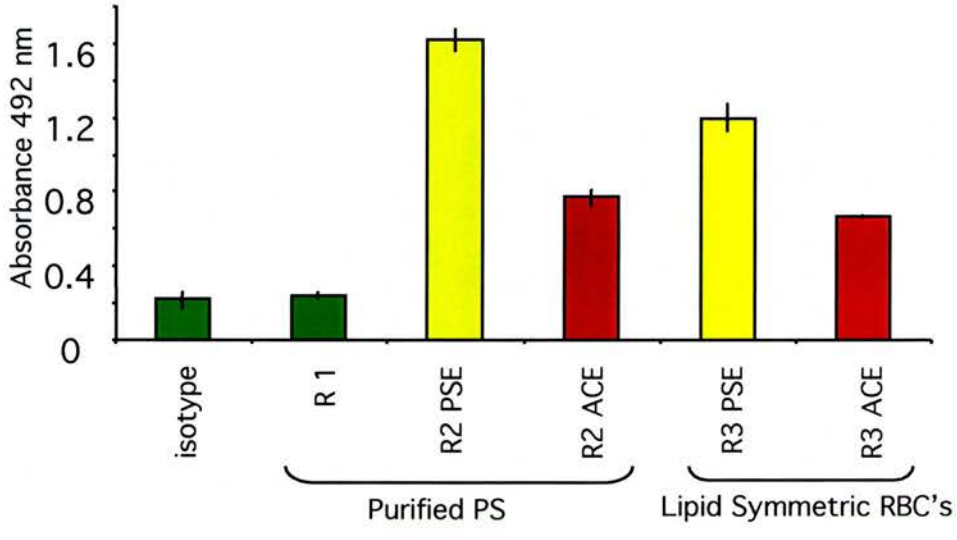


Figure 4-14. Comparison of the binding of polyclonal phage from selections on lipid-symmetric red blood cells (RBCs) and purified PS in an ELISA for PS. Bacterial culture supernatants containing phage were prepared from the first and second round of selections on purified PS (R1 and R2 respectively) and lipid symmetric red blood cells (RBCs - R3) that had been eluted with either PS in solution (PSE yellow bars) or apoptotic cells in solution (ACE red bars). Supernatants were analysed for binding to PS by ELISA. A decrease in binding was seen following selection on lipid symmetric red blood cells. The isotype supernatant represents background binding. Results shown are the mean +/- S.D. of duplicate wells.

phage was seen (from ~300 screened) compared to those selected from the first two rounds on immobilised PS.

The findings of this section can be summarised as follows,

- Phosphatidylserine can be directly bound to gamma-irradiated polystyrene microtitre plates for the specific detection by Annexin V.
- Phosphatidylserine in this format is a suitable tool for selecting phage antibodies capable of specifically recognising the phospholipid in an ELISA.
- Specific elution of phage following a selection provides a means to rapidly amplify phage that bind to the target.
- Immobilised purified phosphatidylserine does not appear to target the selection of phage antibodies towards phospholipids in the way they are found on the cell surface.

Similar to the work described in section 4.2, it seems that purified antigens do not yield high affinity cell-binding phage antibodies for the target molecules of interest to this thesis. Given that purified forms of these targets were employed for selections following the difficulties encountered when using whole apoptotic-cells, the decision was made to explore the use of purified preparations of membrane fractions from apoptotic cells as a selection tool.

4.3 Selection Using Liposomes Prepared from the Membrane Fraction of Apoptotic Cells

4.3.1 Introduction

The results from sections 3.1.4 and 3.1.5 highlighted two of the main problems associated with using whole apoptotic cells for selection. These were (1) loss of apoptotic-cell surface epitopes during the washing process and (2) loss of membrane integrity during the selection incubation period and washing steps allowing phage to bind abundant epitopes found intracellularly. The results from sections 4.1 and 4.2 showed the importance of conformation, context and purity when using immobilised molecules to select for antibodies capable of binding cell-associated epitopes. In addition it is not known whether an ICAM-3 fusion protein, even if produced in cells undergoing apoptosis, could ever model the "apoptotic ICAM-3" found in the context of an apoptotic-cell membrane or whether the purification process itself may affect antibody binding properties.

The results in this section document an attempt to overcome these problems by using liposomes prepared from the membrane fraction of apoptotic cells as a device for selection. Such preparations (also known as proteoliposomes) were employed by Brown et al. for investigating membrane changes occurring during apoptosis. In their system, proteoliposomes derived from primary human neutrophils (polymorphonuclear leukocytes, PMNs) were used as a tool to probe for the constitutive cleavage of membrane-associated actin during spontaneous apoptosis (Brown, Bailey et al. 1997).

For the studies described herein, Mutu I and primary PMNs were used as the source of apoptotic-cell membrane as their recognition by HMDM has been well characterized *in vitro* (Flora and Gregory 1994). Thus, it was possible to isolate monoclonal phage antibodies capable of binding to proteoliposomes in an ELISA format. These clones were analysed for their ability to bind intact apoptotic cells.

4.3.2 Results

4.3.2.1 Immobilisation of proteoliposomes on gamma-irradiated polystyrene.

Proteoliposomes were prepared from the cell lysates of apoptotic Mutu I cells and PMNs by sequential centrifugation to isolate the membrane-containing fraction. Competence to bind Annexin V in an ELISA format was used to demonstrate that the proteoliposome solutions could be passively adsorbed to polystyrene. Wells of microtitre plates containing proteoliposomes prepared from both Mutu I cells and PMNs showed comparable Annexin V binding to wells containing PS liposomes figure 4-15.

As proteoliposomes were prepared in the absence of detergent (i.e. membrane lipids would not be partitioned from other membrane components) the presence of PS indicated that such a cellular fraction immobilised in this way would provide a reasonable representation of membrane derived from apoptotic cells.

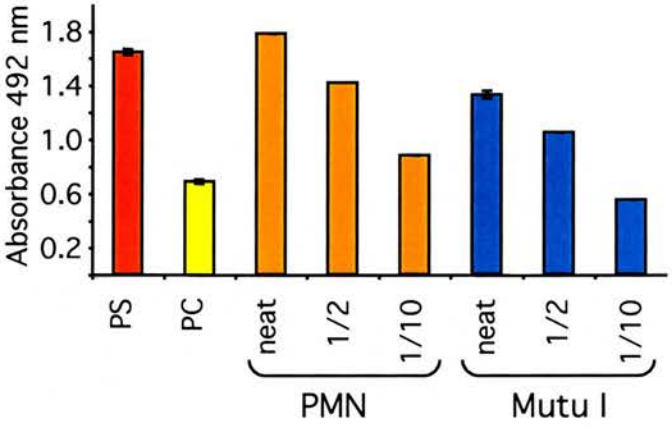


Figure 4-15. Adsorption of liposomes prepared from apoptotic-cell membranes to polystyrene as detected by Annexin V ELISA. Plates were coated with proteoliposome preparations derived from apoptotic primary human neutrophils (PMN) or the B-cell lymphoma cell line (Mutu I), phosphatidylserine (PS) or phosphatidylcholine (PC) by incubation for 45min at 37°C. Solutions were prepared and diluted in serum-free HBSS . $Absorbance_{492\ nm}$ represents optical density values corresponding to proteoliposome adsorption after detection with Annexin V. Results shown are the mean +/- S.D. of duplicate wells. Representative of proteoliposomes prepared on three separate occasions.

4.3.2.2 Selection of immobilised proteoliposomes derived from apoptotic-cell membranes

Initially, panning was performed using proteoliposomes from apoptotic Mutu I cells. Following two rounds, the enrichment of phages specific to components of the selection material was assessed by ELISA (Figure 4-16 panel A). Polyclonal preparations were tested for their ability to bind PS and PC (representing common membrane components) in addition to Mutu and PMN proteoliposomes. BSA was included as a negative control. The general trend was an increase in binding following the second round of selection. A modest signal for both PS- and PC-coated wells indicated that a subpopulation of clones were able to bind lipids. An equi-molar concentration of solutions was used to coat the plastic (although this may not reflect the absolute level of lipid that remains bound). Despite the inclusion of steps to pre-deplete eluted phage on PC coated tubes, a similar-strength signal was observed for both PC and PS.

A signal was observed for both proteoliposomes prepared from PMNs as well as Mutu I cells (Figure 4-16 panel A). This suggests selection toward antigens common to both cell types. Even though proteoliposomes for each cell type were prepared using the same number of cells, a slightly higher signal was observed for proteoliposomes prepared from PMNs compared to Mutu I cells (Figure 4-16 panel A). This may represent either a higher concentration of total antigen in the PMN preparation (because of their inherent ability to maintain membrane integrity during apoptosis) or that each PMN cell expresses a higher level of the antigens specific for

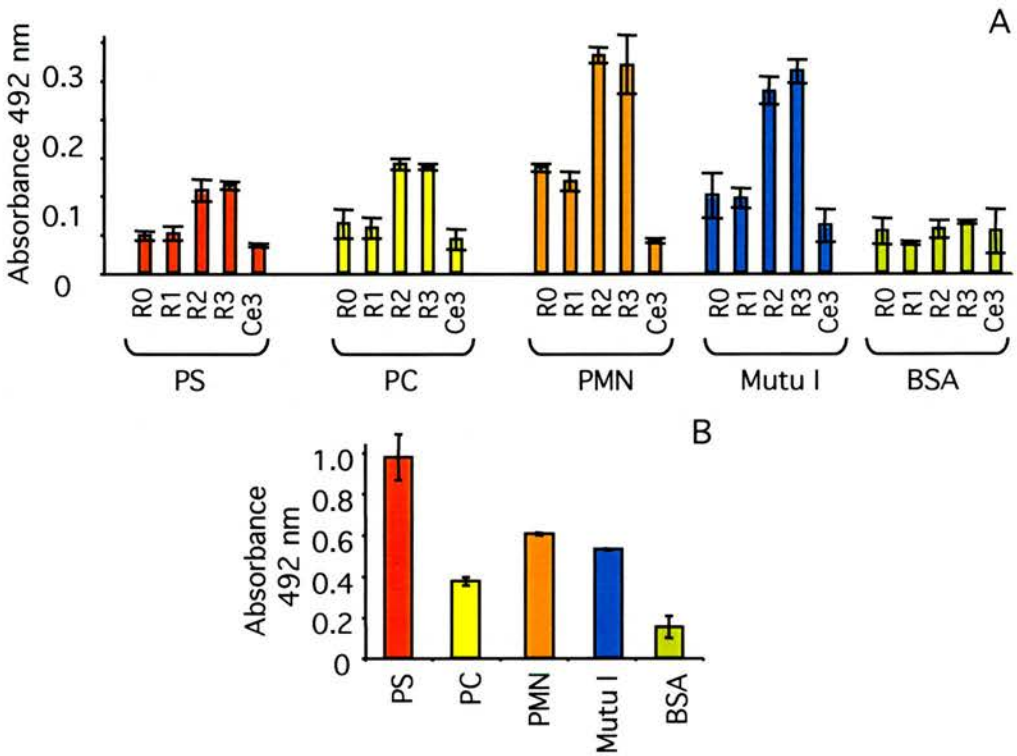


Figure 4-16. Following 2-3 rounds of panning on apoptotic-Mutu derived proteoliposomes, enrichment of phages specific to components of the selection material was observed.

A Bacterial culture supernatants containing phage were prepared from various rounds (RO to R3 respectively) of selection on proteoliposome-coated immunotubes, and analysed for binding to PS liposomes, PC liposomes, proteoliposmones derived from PMNs (PMN) or Mutu I cells (Mutu I), or BSA as an irrelevant antigen. Ce3 phage antibody supernatant (α-CD14) represents background binding. Results shown are the mean +/- S.D. of duplicate wells. **B** Adsorption of proteoliposomes to wells was quantified using Annexin V.

the selected phages. A slightly stronger signal for Annexin V binding to the PMN preparation (Figure 4-16 panel B) supported the former suggestion.

In an attempt to enrich further for antigen-binding clones, a third round of selection was performed using PMN proteoliposomes. The rationale for changing cell types as an antigen source was to try and lose common B-cell specificities whilst still providing common apoptotic-leucocyte epitopes. After the third round, no significant increase in binding to any of the antigens tested was seen. (Figure 4-16 panel A) Consequently the decision was made to screen individual clones from the second and third rounds for their ability to bind proteoliposomes by ELISA.

4.3.2.3 Screening of monoclonal phage

~80 clones from a total of ~1500 tested bound to apoptotic-Mutu I derived proteoliposomes in ELISA format. Of the ELISA-positive clones, 6 preferentially bound to the "late apoptotic" sub-population of an ionomycin-treated Mutu culture. However, as was previously encountered for cell-binding phage from selections on immobilised PS, these clones failed to bind cells following regrowth on a larger scale despite maintaining phage concentration and ELISA reactivity (figure 4-17).

Explanations for the loss of cell binding capacity, such as differences in avidity of phage clones between batches (i.e. batches of lower avidity which maintain an affinity suitable for ELISA but insufficient for flow cytometry), will be discussed in more detail in section 5.

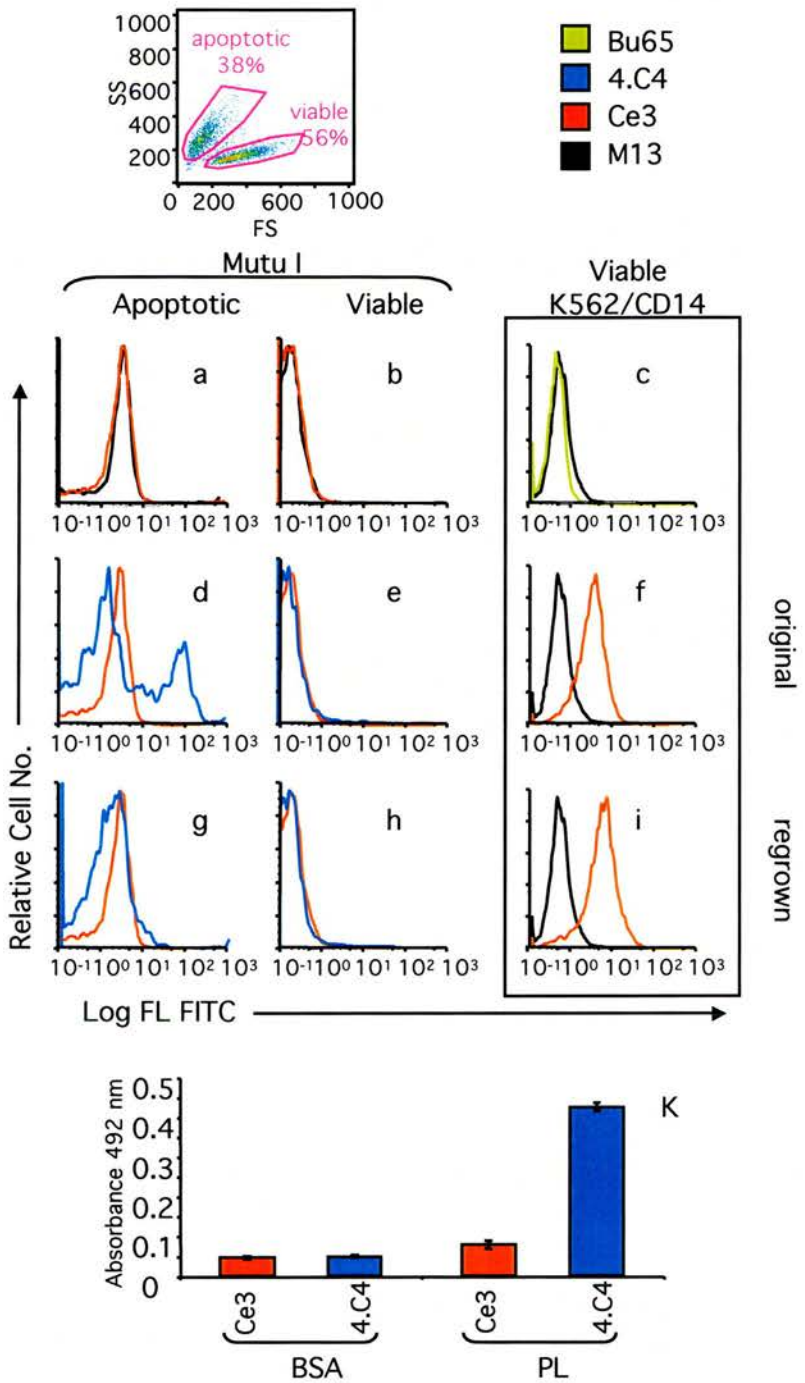


Figure 4-17. Analysis of regrown phage antibodies from selections on apoptotic-Mutu derived proteoliposomes as determined by flow cytometry and ELISA. Individual phage clones were prepared from culture stocks originally identified as positive (e.g. clone 4.C4) for reactivity towards apoptotic cells. Original (d,e) and regrown (g,h) batches of clone 4.C4 were analysed for the ability to bind apoptotic cells by flow cytometry. Clone 4.C4 lost the ability to bind cells following regrowth. Clone Ce3 (anti-CD14) included as a control during production of all batches maintained binding to CD14 expressing cells (f,i). K, regrown monoclonal preparations were also analysed for binding to proteoliposomes or BSA as a control. Clone 4.C4 maintained ELISA reactivity following regrowth. Results shown are the mean +/- S.D. of duplicate wells. Data is representative of two independent analyses.

The work described in this section can be summarised as follows;

- Proteoliposomes derived from the membrane fraction of apoptotic cells were prepared and their passive adsorption to a polystyrene support established.
- Proteoliposomes immobilised in this manner can be used to select a population of antibody fragments with affinity for immobilized PC, PS and proteoliposomes in an ELISA format.
- A small number of antibody fragments from these selections that bound antigens in an ELISA also bound preferentially to a sub-population of late apoptotic cells within an induced culture.
- None of the monoclonal phage retained cell-binding capacity following regrowth. Loss of reactivity towards cells was not due to a decrease in total phage concentration.

5 Discussion - bacteriophage display work

The aim of the work documented in results chapters 1 and 2 was to select antibody fragments with specificity for apoptotic cell-associated epitopes from a bacteriophage antibody library. Selections were targeted towards whole cells overexpressing ICAM-3, purified preparations of recombinant ICAM-3, purified phosphatidylserine (PS) and the purified membrane fraction of apoptotic cells. Several methods were employed in an attempt to separate clones that bind from those that do not. Novel protocols for selections were designed and optimised by modification of previous methods performed using this library and successful published examples.

5.1 Summary of findings

Results chapter 1 began by describing the development of techniques aimed at obtaining antibody fragments to the form of ICAM-3 found on apoptotic-cells ("apoptotic ICAM-3") by selecting and screening on whole cells, exogenously overexpressing ICAM-3. Following optimisation of phage antibody selection using viable K562 cells in suspension (section 3.1.3), a repertoire of many different cell-binding phages was isolated using apoptotic K562 cells overexpressing ICAM-3, demonstrating functionality of both the phage antibody library and the basic techniques for its' manipulation.

However, none of the identified cell-binders proved to be specific for ICAM-3 or apoptotic cells. What is more, the clones selected for all seemed to bind epitopes expressed at higher levels on viable cells (section 3.1.4).

As a result, an alternative approach to reduce the selection of phage that bind to unwanted targets was sought. Negative cell-selections have proven an inefficient means to remove unwanted phage (Van Ewijk, de Kruif et al. 1997; Topping, Hough et al. 2000) and introduce the risk of losing phage of interest through non-specific binding to the "negative" antigen. Therefore, the decision was made to try dual positive and negative selection where a small number of ICAM-3-positive apoptotic cells were mixed with a larger number ICAM-3-negative viable cells. However these more sophisticated selections, aimed at homing in on "apoptotic ICAM-3" in a single selection procedure were also unsuccessful (section 3.1.5). This was reflected by the lower number of clones returning from sequential rounds of selection than had been observed for K562 selections (section 3.1.3 and 3.1.4), suggesting that there may have been a fundamental flaw in this procedure (although it is difficult to assess the value of separate methods without comparing directly to positive cell selections using the same cell type performed in parallel). Thus, the results presented in results chapter 1 suggested that the use of whole apoptotic cells for selection would not provide a simple means to achieve the aims of this thesis. This may in part have been due to the availability on whole cells of a wealth of alternative antigens (carbohydrates, lipids and proteins) in addition to the sought targets. These molecules will be common to other cells, therefore during selection the type of antigens expressed on the cell surface are likely to bias the type of antibodies selected from the phage library (discussed in more detail in section 5.3).

In appreciation that many successful results of phage display have been achieved using selections carried out on purified antigens (Dekruif, Boel et al. 1995; Hodits, Nimpf et al. 1995; Pierce 2000; Horn, Nielsen et al. 2003), the work described in

results chapter 2 attempted to overcome the problems mentioned above by using purified forms of apoptotic-cell-associated targets, namely, ICAM-3, phosphatidylserine and membranes prepared from apoptotic cells. Targeting selection to ICAM-3 in the way it is found on cell-surfaces using immobilised recombinant ICAM-3 proved difficult as epitopes present in purified preparations of the protein, other than those specific to ICAM-3, dominated selections (section 4.1). However, selections on purified PS demonstrated the functionality of the phage library with regards to the ability to select for immobilised-lipid-specific phage (section 4.2), and that the specific elution approach taken to achieve this can work as a quick way to amplify phage that bind to the desired target. However, relatively few of these clones bound to apoptotic cells, and of those that did, their affinity for cells was relatively low (a general feature of phage antibodies selected from unbiased libraries, see section 5.2).

Given that immobilisation of antigens to a particular surface might favour exposure of only certain parts of the antigen, there was never any guarantee that the epitope(s) on ICAM-3 or PS associated with their function in the lipid bilayer of apoptotic cells would be represented in a purified, immobilised form (c.f. introduction section 1.2.5.2.1.1). For these reasons the decision was made to use liposomes prepared from the membrane fraction of apoptotic cells as a device for selection. However none of the minority of proteoliposome positive clones reproducibly bound to apoptotic cells following growth on a larger scale.

The issues encountered in trying to realise the aims of this work using phage display can be summarised as;

- Technical difficulties associated with the use of bacteriophage antibody libraries (especially of naïve origin).
- Difficulties of using whole cells as a selection agent for phage display, a problem that is confounded by the fragility of apoptotic cells.
- Unfavourable properties of immobilised antigen as a target for selection.

The discussion will now focus on what these findings mean, how they compare to published/previous phage work and the direction which future work with these techniques could be taken.

5.2 General difficulties of working with naïve bacteriophage antibody libraries

Phage-display technology offers the potential to generate antibodies specific for any epitope. Statistically speaking, the higher the complexity of the library, the greater the chance is that it will contain antibodies of the desired specificity. However, high-complexity libraries have their downside for *in vitro* use. For example, in the case of the library used for this work, which has an estimated complexity of 3.8×10^8 specificities, each clone would only have been represented ~1500 times during a selection (i.e. $\sim5\times10^{11}$ input particles per selection ÷ 3.8×10^8). Furthermore, as a result of the phagemid selection system, the antibody coat protein fusion construct competes with the coat protein from the helper phage for incorporation into phage

particles. As a result only some particles display antibody fragments, which reduces the number of functional phages 10 fold or more (Breitling and Dèubel 1999). During selection, few specific phage particles exist bound to their antigen (i.e. some exist freely in solution), lowering even further the probability that clones survive the first round of selection. Thus, one reason for explaining the consistent inability to isolate an ICAM-3 antibody is to hypothesise that there are very few ICAM-3 specific antibodies within the phage library that was used, which in combination with the small likelihood of binding during the first round of selection, resulted in their loss from subsequent sub-libraries. This is, however, unlikely considering the successes with this library for similar targets (Dekruif, Boel et al. 1995; Pierce 2000). Alternative explanations will be discussed in sections 5.5 and 5.6 below.

Phage antibody technology using naïve libraries does not have the advantage of the body's natural affinity maturation process whereby antibodies mutate over relatively short periods generating higher affinity for their targets. This drawback may explain why only low-affinity antibodies to phosphatidylserine were obtained following the specific elution approach employed for selections against this phospholipid (section 4.2). One way to obtain antibodies of higher affinity by this approach could be to use longer incubation during the elution step to remove antibody fragments with longer "on rates" or to use a more severe elution step (low pH). Alternatively, the affinity of the selected antibodies could be improved by mutation *in vitro* (Gram, Marconi et al. 1992).

Despite the shortcomings mentioned above, the quality of the antibody library was assessed in the very first section and was shown to contain cell-binding phage of relatively high affinity (section 3.1.3). Direct selection from the library using apoptotic cells led to the selection of irrelevant cell-binding antibodies indicating the requirement for extensive depletion (section 3.1.4). However, dual positive and negative selections failed to enrich even for common cell-binding phage (section 3.1.5). Another option to prevent enrichment of undesirable clones would be to switch cell lines during the panning procedure or to use purified proteins in between selections on cells.

5.3 Additional problems imposed by apoptotic cells

All cell-binding monoclonal phage antibodies that were identified from screening of the selections carried out on apoptotic K562/ICAM-3 cells showed a much lower signal on apoptosis-induced populations than viable on populations (section 3.1.4). This suggests that the antigens selected for were present at lower levels on cells as a result of programmed cell death. Indeed, a number of macromolecules (including ICAM-3)(Moffatt 1999) are known to be lost from the surface during apoptosis (Brown, Kluck et al. 1996; Hart, Ross et al. 2000). Additionally, even in cell cultures provided with a uniform dose of apoptotic stimuli, heterogeneity with respect to the stage of apoptosis is often observed amongst a population, such that cells with a compromised plasma membrane could have been amongst the selection mixture, or have arisen during the washing procedure. The release of degradative enzymes from such "leaky cells" could cleave antibody fragments from phage particles or could have modified the target (ICAM-3) making further propagation

impossible. The addition of protease inhibitors to apoptosis-induced cultures could potentially limit such harmful effects. Loss of "apoptotic-target" specific phage could also have occurred through loss of cellular material (presumably with phage bound to them) in the wash supernatants, as for the selections on apoptotic K562 cells during these selections. A substantial decrease in cell-pellet size during the washing process was observed, a phenomenon that was not seen during viable cell selections (section 3.1.3). Furthermore, as permeabilised cells display a generalised increased "background" binding of antibodies (phage or otherwise), this could have led to the propagation of unwanted clones. A more detailed assessment of cell viability following selection would be worthwhile in the future.

With these drawbacks in mind, attempts were made to select for apoptotic-cell markers using purified preparations of antigens. However, this approach also proved to have significant shortcomings.

5.3.1 Problems associated with the use of purified antigens for selection.

5.3.1.1 Quality of antigen

It is well known that the quality and quantity of the target antigen are important parameters when selecting from highly complex antibody libraries (Breitling and Dèubel 1999). Thus, the quality of the selecting antigen determines the properties of the selected antibodies. Section 4.1.3.2 showed the difficulties encountered obtaining a uniform preparation of ICAM-3. Such "purified preparations" were shown to contain large amounts of protein in addition to the target antigen, despite measures such as growth under serum-free conditions (section 4.1.3.2.2), indicating a

possible preferential enrichment of phage that bind to contaminating components of the ICAM-3 preparations. Possible ways to overcome this would be to include an isotype fusion protein in the selection mixture or to use more extensive purification procedures (e.g. liquid chromatography) during the preparation of the antigen. However purification procedures could very well, and may indeed have had effect on the antigens binding properties by altering their conformation.

5.3.1.2 Epitope dominance and immunogenicity of ICAM-3

Due to the requirement for multiple rounds of selection to enrich and isolate specific antibody fragments, phage display selections will generate clones with specificity for a dominant epitope either on the target molecule itself or within the antigen mixture. Repeatedly choosing clones with the highest binding affinity or which have a growth advantage in this way can lead to loss of diversity amongst potential binders. This may explain the apparent impediment for selection of phage against ICAM-3, that is, if the available ICAM-3-specific antibodies within the library are of relatively low affinity or if (as was realised in section 4.1.3.2) ICAM-3 represents a minor component of purified preparations/cell-surface.

It is also possible that ICAM-3 offers only "weak epitopes" for *in vitro* selections. Experts in the phage display field have published evidence that the type of epitopes on a target antigen can have profound effects on the success of isolating phage antibodies. For example, using the same methodology and starting with the same library, Hoogenboom *et al.* were able to select an exceptionally high frequency of antigen-specific phage antibodies to CD36. However, none were obtained to a somatostatin receptor (sst), even though both targets were overexpressed to the same level on the same cell type. Even following attempts to extensively pre-adsorb the

library with sst-negative cells or specific elution with somatostatin, no success was achieved (Hoogenboom, Lutgerink et al. 1999). The authors of this work concluded that glycosylation of the cell-surface-exposed portion of sst prevented enrichment of specific phage. Comparable difficulties were encountered in trying to isolate phage antibodies specific for the glycoprotein, Mucin-1 (Muc-1). Following numerous selection approaches and the screening of thousands of clones by ELISA, only one binder was identified which upon subsequent analysis failed to bind cells expressing Muc-1 (personal communication, Mark de Souza, Dyax Corp). In a similar way to Muc-1, ICAM-3 is the most heavily glycosylated member of its protein family. Therefore, quite feasibly, the glycosylation on domains 1 and 2 of ICAM-3 could prevent phage from accessing the peptide stalk of the molecule. Whilst appreciating that the phage library used in these studies is not peptide specific, masking of peptides would mean the only available ICAM-3-specific sites would have been the carbohydrate chains. Although phage display has been used to isolate carbohydrate-binding antibodies (Mao, Gao et al. 1999), glycoconjugates are conformationally very dynamic, a property which reduces the effective concentration of target antigen at any one time and yields only low-affinity antibodies which could easily have be overlooked during primary screening. Therefore, any selection procedure using glycosylated domains 1 and 2 of ICAM-3 could be problematic for this reason. To test the hypothesis that ICAM-3 provides only "weak epitopes" for use in phage-display one could compare selections carried out in parallel on deglycosylated ICAM-3 and the fully glycosylated protein.

Numerous other approaches aimed at maximising the targeting of selections towards ICAM-3 were considered, but due to time restraints, not attempted during this work.

For example, an immune phage antibody library focused on ICAM-3 could be constructed (produced from an IgG library from animals immunised with ICAM-3). An interesting approach, named "ProxiMol®technologies", developed by "Cambridge Antibody Technology group", allows focusing of selections to minor components of an antigenic mixture (http://www.biotechsforum.com/cat.htm). The strategy taken is to biotinylate only the phage bound to the target by peroxidase catalysis of the biotinylation reaction with a pre-existing HRP-conjugated antibody that binds to a nearby site on the antigen. Streptavidin can then be used to separate target-specific phage from phage bound to other antigens. This technique would be feasible for domain 1 ICAM-3 (the site recognised by antibodies which lock apoptotic cell recognition), as a number of epitope mapped antibodies to this molecule exist (Moffatt 1999).

5.4 Many clones which screened positive by ELISA failed to bind cells

For selections carried out on His-tagged ICAM-3 (section 4.1.3.2), none of the phage clones that screened positive by ELISA demonstrated cell binding. For these selections magnetic separation was used to isolate phage that bind to the immobilised antigen during the wash steps. This avoided the strong shear-forces either associated with other washing techniques or what would be experienced during flow cytometry. This may have selected phage clones which, although able to express antigen-specific fragments, may have off-rates that are higher than the threshold set by the cell-screening but lower than set by the ELISA. One way to increase the overall avidity of weak affinity phage preparations is by increasing the number of antibody

fragments presented on phage particles by using a helper phage named "hyperphage" (Rondot, Koch et al. 2001). Hyperphage have been shown to increase the antigen-binding activity of phage preparations by ~400 fold.

In addition to selections carried out on His-tagged ICAM-3, numerous ELISA-positive-PS-specific phages from selections on the purified phospholipids also failed to bind apoptotic cells (section 4.2.2.2.2). Strong shear forces were used for washing antigen-bound immunotubes following these selections, arguing against selection of very low-affinity clones. Therefore, it is conceivable that immobilisation of the target antigens to a hydrophobic surface may have exposed hydrophilic areas to the phage, or may even have denatured the targets such that they no longer resembled the cell-associated counterparts. To prevent this phenomenon, selections could be carried out with tagged antigens in solution followed by capture of the antigen-phage complex prior to washing (Goletz, Christensen et al. 2002).

5.5 Lack of reproducibility in cell-binding following regrowth of phage clones

Many of the apoptotic cell-positive phage obtained following selections carried out on phosphatidylserine and proteoliposomes, (sections 4.2.2.2.2 and 4.3.2.3 respectively) failed to bind cells following regrowth on a larger scale. Considering that the original batches of these clones were observed to bind cells when staining was carried out on different occasions it is unlikely that these were false positives. Although it is possible that the true positives were outgrown by antigen-negative phage contaminating the "monoclonal" cultures, it could also be that subtle mutations to the genes encoding antibody fragments occurred. Bacteriophage

genomes exhibit a high degree of dynamic instability (Palevitz 2003). It is also possible that selection of phage specific for molecular patterns on apoptotic cells could have provided a driving force for selection of low affinity phage or spontaneously arising mutants during re-growth. The rationale for this is discussed in depth in the following section.

5.6 Depletion of high affinity apoptotic-cell-associated-pattern-specific antibodies from the phage library by pathogen-associated-molecular patterns.

Many of the receptors used by phagocytes to interact with apoptotic cells are known to recognise microbial components (see introduction section 1.2.6) eluding to the likelihood that the molecular patterns associated with apoptotic cells (ACAMPs) resemble those found on microbes (PAMPs). One might even propose that the glycoconjugates of ICAM-3, or more specifically "apoptotic ICAM-3", bear resemblance to microbial moieties. The fact that the pattern-recognition-receptor (PRR), dendritic-cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) a receptor for ICAM-3, also binds many pathogen-associated structures (Maeda, Nigou et al. 2003) would support this. Given that bacteriophage libraries are propagated in bacterial cultures, clones displaying antibody fragments with high affinity for epitopes resembling PAMPs or ACAMPs could provide a growth impediment to bacterial hosts or even be absorbed out of solution. If this were the case, clones specific for bacterial components that resemble ACAMPs may have been under-represented in the initial phage library, or subtle mutations to regions of a phage genome encoding antigen-binding sites that result in a lower affinity for bacterial

host components and a consequent growth advantage to the phage would dominate. Mutations of this kind resulting in subtle differences to the antigen binding properties of the antibodies may explain why some clones lost cell-reactivity but retained binding of immobilised antigen. Consistent with the hypothesis that ACAMPs resemble PAMPs in three-dimensional space, the very antibody used for detecting bacteriophage throughout this work was shown to bind a proportion of apoptotic eukaryotic cells in preference to viable cells (figure 5-1).

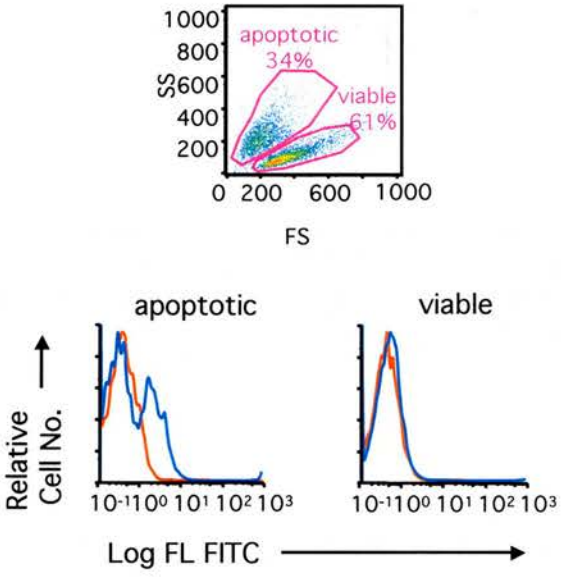


Figure 5-1. Preferential binding of anti-M13 bacteriophage antibody to apoptotic cells as assessed by flow cytometry. Mutu I cells were induced to undergo apoptosis by treatment with ionomycin for 16 hours. The ability of an antibody to the major coat protein of M13 bacteriophage (blue histograms) to bind cells was compared to binding of isotype-matched antibody (red histograms) by flow cytometry. Cells at a density of 2.5×10^6/ml were incubated with antibodies at a concentration of 25μg/ml. After washing, bound antibody was detected with FITC labeled goat anti-mouse secondary antibody. Within the population cells were defined as either apoptotic or viable on the basis of light scatter properties (see methods section 2.3.3.3)

5.7 Conclusion to phage-displayed antibody studies

From the position reached in this work, there remains potential for using phage antibody approaches to isolate antibody fragments with specificity for apoptotic-cell-associated targets. Several possible areas for improving on the limited success seen in these studies include;

- Sub-cloning of the DNA encoding the antibody-binding site from phage into a non-bacterial expression system immediately subsequent to identification of apoptotic-cell reactivity and prior to loss of cell-binding capacity.
- Improved purification of target antigens (for example by chromatographical methods) to reduce levels of unwanted epitopes in selections.
- *In vitro* affinity maturation of low-affinity target-binding clones.
- Creating a library of patient-derived repertoires from individuals with autoimmune diseases linked to the presence of persistent apoptotic-cells (or from a similar mouse model) to increase the frequency of target-specific clones in a phage library.

However, in light of the apparent impediment for isolating desired antibodies from phage propagated in bacterial species the decision was made to investigate further whether apoptotic-cell-associated molecular patterns indeed resemble pathogen-associated-molecular patterns.

6 Results Chapter 3: Probing Apoptotic Cells for Ligands of Pattern Recognition Receptors using anti-Microbial Antibodies.

6.1 Introduction

Results chapters 1 and 2 document attempts made to isolate phage antibodies specific for apoptotic cells. With the knowledge that many pattern recognition receptors of the innate immune system recognise both apoptotic cell and microbial structures (ACAMPs and PAMPs), it was hypothesised that the limited success seen with the phage approach may in part have been due to the fact that a bacterial system was used to propagate phage antibody library. That is, high affinity antibodies to ACAMPs may have been "negatively selected" from the library by virtue of their similarity to PAMPs. Thus, the work presented in this chapter aimed to test the hypothesis that antibodies which react with PAMPs can cross-react with ACAMPs. This was tested by screening a panel of mAbs raised against microbial-associated CD14 ligands for reactivity towards apoptotic cells with the intention of characterising the epitopes bound by any antibodies filling this criterion and testing such antibodies for their ability to block the recognition of apoptotic cells by macrophages.

6.2 Results

6.2.1 Some antibodies previously characterised as having specificity towards lipopolysaccharide also bind to apoptotic cells.

Initial results obtained from screening a panel of antibodies raised against pathogen-associated epitopes for their ability to bind apoptotic Mutu I cells are summarised in table 6-1. Apoptotic cells were stained by 3 of 13 mAbs tested. Figure 6-1 displays example flow cytometric histograms of such staining. Reactivity with mAb WN1 222-5 was the most marked. This antibody has been characterised as having broad cross-reactivity towards numerous forms of rough and smooth LPS (Di Padova, Brade et al. 1993). Recent studies demonstrated that mAb WN1 222-5 binds to the inner core region of LPS at a position most distal to Lipid A and have defined the structural element required for its recognition (Muller-Loennies, Brade et al. 2003).

6.2.1.1 Staining of apoptotic cells with anti-LPS mAbs is not cell-surface restricted.

Confocal microscopic analysis was used to examine the distribution of immunofluorescence staining with the apoptotic-cell-reactive α-LPS mAbs. This revealed staining throughout the cytoplasm of apoptotic Mutu I cells suggesting recognition of intracellular epitope(s) made accessible following the loss of membrane integrity as a result of apoptosis. All three apoptotic-cell-positive mAbs exhibited a similar staining pattern of the cytoplasm. Figure 6-2 shows staining with

Ab Name	Pathogen Specificity	species	AC reactive	Supplier
wn1 222-5	**LPS**	mouse	+	EU
R1	**R1 LPS**	mouse	-	EU
R2	**R2 LPS**	mouse	-	EU
R3	**R3 LPS**	mouse	weak +	EU
F6 446-24	**LPS**	mouse	-	EU
F6 504-11	**LPS**	mouse	+	EU
F6 514-12	**LPS**	mouse	-	EU
FK4 224.10 10	**LPS**	mouse	-	EU
FK4 224-14	**LPS**	mouse	-	EU
FK4 224-17	**LPS**	mouse	-	EU
FK4 224-24	**LPS**	mouse	-	EU
UT	**LPS**	mouse	-	EU
AD 1	**Chlamydial LPS**	mouse	-	BU
MOMP no 1	**Chlamydial MOMP**	goat	-	EU
MOMP no 2	**Chlamydial MOMP**	mouse	-	BU
Ad x	**coat protein Ad5**	mouse	-	EU
Ad 45	**coat protein Ad5**	mouse	-	EU

Table 6-1. Panel of antibodies with specificity for pathogen associated epitopes included in a preliminary screen for the ability to bind to apoptotic cells. Antibodies with a known specificity for pathogens were tested for the ability to bind apoptotic Mutu I cells by immunofluorescence and flow cytometry. Antibodies were incubated with apoptotic cells at concentrations previously established for labeling of their pathogen-associated epitope. Of 13 antibodies to LPS tested, 3 (indicated by + as AC reactive) produced a significant signal. Neither of the 2 antibodies to viral coat proteins or 2 antibodies to bacterial major outer membrane protein (MOMP) produced a signal. EU; Edinburgh University, BU; Birmingham University, LPS; lipopolysaccharide.

monoclonal antibody WN1 222-5 as an example. In contrast, no staining was seen with control mAbs (Fig 6-2 panels D, E).

Having established that some antibodies to LPS display binding to apoptotic cells, the decision was made to locate a source of α-LPS mAbs with similar AC cross-reactivity in order for these studies to be extended further as those obtained from Edinburgh University were in limited supply. Antibodies were selected on the basis of their previously characterised ability to cross-react with the broadest range of LPS species. Such antibodies are often obtained by immunisation with rough mutants of bacteria which expose conserved inner-core LPS determinants. Indeed antibodies raised against the rough mutant, *Escherichia coli* O111:B4 (strain J5) and affinity purified against LPS derived from *Salmonella* were shown to react with a series of unrelated Gram-negative bacteria (Tyler, Spears et al. 1991). The same *E. coli* strain, in addition to other species lacking the hypervariable O-antigen of LPS such as *Chlamydia trachomatis*, were used by the antibody manufacturers at QED Biosciences Inc for an immunisation. The resultant commercially available panel of mAbs are marketed as having broad cross-reactivity to LPS.

Table 6-2 lists the antibodies chosen from those supplied by QED Biosciences Inc along with their reported LPS reactivities. Specificity of these antibodies to LPS was later confirmed by ELISA (see appendix A6-1). Staining of apoptotic Mutu I cells with the three chosen antibodies by concentration-dependent titration was assessed by flow cytometry (Figure 6-3) All three antibodies preferentially stained apoptotic cells compared to control Abs and no staining was observed for viable cells (top three panels, green histograms). The strongest signal was seen with mAb 15308, which showed a significant shift in fluorescence at 0.78 μg/ml.

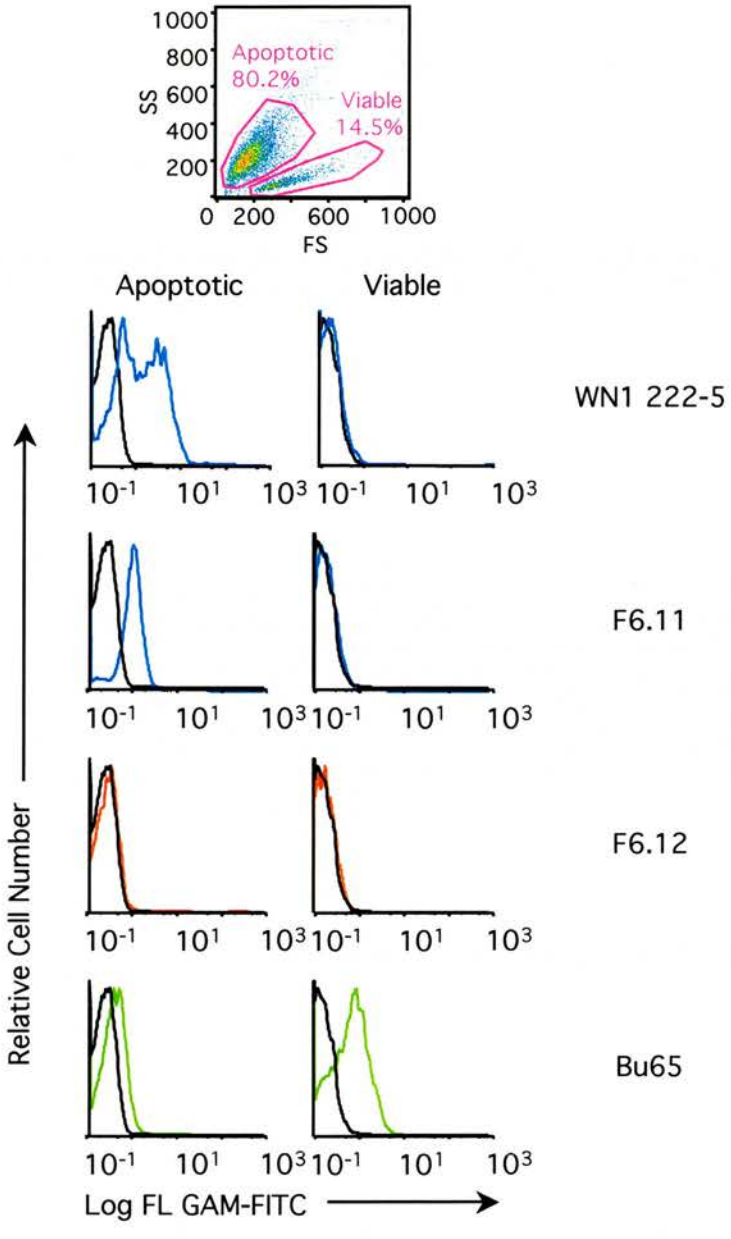


Figure 6-1. Some antibodies previously characterised as having a specificity for lipopolysaccharide also bind to apoptotic cells. Antibodies with a known specificity for LPS were tested for their ability to bind apoptotic Mutu I cells by flow cytometry. Mutu I cells were treated with ionomycin for 16h before incubation with primary antibodies. Bound antibody was detected with FITC labeled goat anti-mouse secondary antibody (GAM-FITC) and cell-associated fluorescence was quantified by flow Cytometry.
Upper panel; viable and apoptotic cells were discriminated on the basis of light scattering properties as described in Materials and Methods (section 2.3.3.3). Lower panel; α-LPS antibodies WN1 222-5 and F6.11 (blue histograms) showed a clear signal on apoptotic cells where as other α-LPS antibodies such as F6.12 (red histograms) exhibited no staining compared to secondary antibody alone (black histogram). Green histograms represent staining with Bu65 (α-ICAM-3).

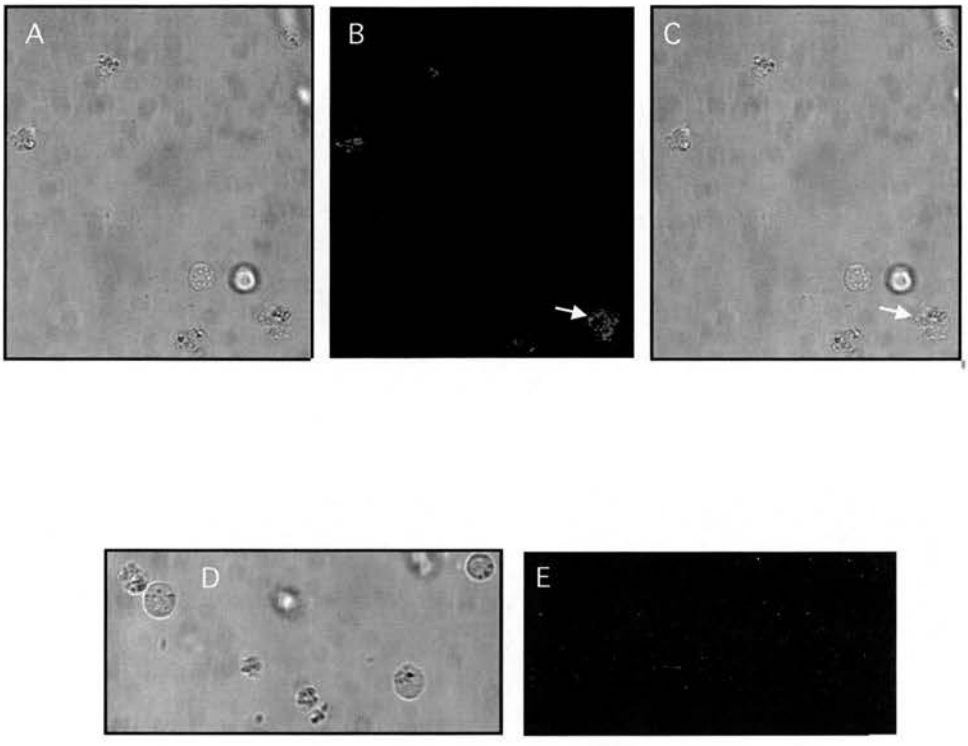


Figure 6-2. Binding of anti-LPS mAbs to apoptotic Mutu I cells is not restricted to the plasma membrane as determined by confocal microscopy. Mutu I cells were treated with ionomycin for 16h before incubation with primary antibodies. Binding of the α-LPS antibody, WN1 222-5 (B,C) or isotype-matched control, γ2a (D,E) was detected with goat anti-mouse secondary antibody labeled with alexaFluor-488 (green). Cell-associated fluorescence was visualised using a laser-scanning microscope. The images represent individual cross-sections taken from three-dimensional serial sections of cells. Staining was observed throughout the cytoplasm but was not apparent in nuclear regions (seen most clearly with the cell to the lower right of panels B and C (arrow)). A & D; bright field images, B and E; fluorescence, C; merge.

Clone name	Marketed LPS Specificity	Species	Isotype
15306	*Shigella sonnei* *Salmonella typhimurium* *Serratia marcescens* *Proteus mirabilis* *Proteus vulgaris* *Acinetobacter calcoaceticus*	mouse	IgG2a
15308	*Enterobacter aerogenes* *Serratia marcescens* *Proteus mirabilis* *Acinetobacter calcoaceticus* *Pseudomonas aeruginosa*	mouse	IgG3
15174	*Chlamydia trachomatis*	mouse	IgG2a

Table 6-2. Reported reactivities of commercially available cross-reactive anti-LPS mAbs used in these studies. Antibodies were assessed for binding to purified preparations of LPS in an ELISA.
LPS of Chlamydia trachomatis lacks the 0-polysaccharide region most distal to Lipid A. Thus, although being marketed as anti-Chlamydia antibody (the most popular from several supplied by QED Biosciences), mAb 15174 was chosen for its potential reactivity towards conserved core regions of LPSs.
(http://www.qedbio.com/alpha_e_g.htm)

Name	Derived from	Culture type	Assessment
293T	Primary human embryonal kidney cells	adherent	AI , FP
MCF-7	Adenocarcinoma of human mammary gland epithelial cells	adherent	AI
HeLa	Human cervical carcinoma	adherent	FP
A549	Lung carcinomatous tissue	adherent	AI , FP
BJAB	Epstein-Barr virus-negative Burkitt's lymphoma	suspension	AI
Mutu I	Epstein-Barr virus-positive Burkitt's lymphoma B-cell (clone 179)	suspension	AI , FP
K-562	Multipotential, malignant hematopoietic cells	suspension	AI , FP
NsO	Mouse myeloma	suspension	AI
A1.1	Mouse thymoma	suspension	AI
PMN	Blood of a healthy human donor	primary	AI
COS-1	Kidney cells of a monkey	adherent	AI , FP

Table 6-3. Cell types with which mAb 15308 has tested positive for binding. All cells either induced into apoptosis (AI) or fixed and permeabilised (FP) have shown to bind mAb 15308 as assessed by flow cytometry and/or microscopy.

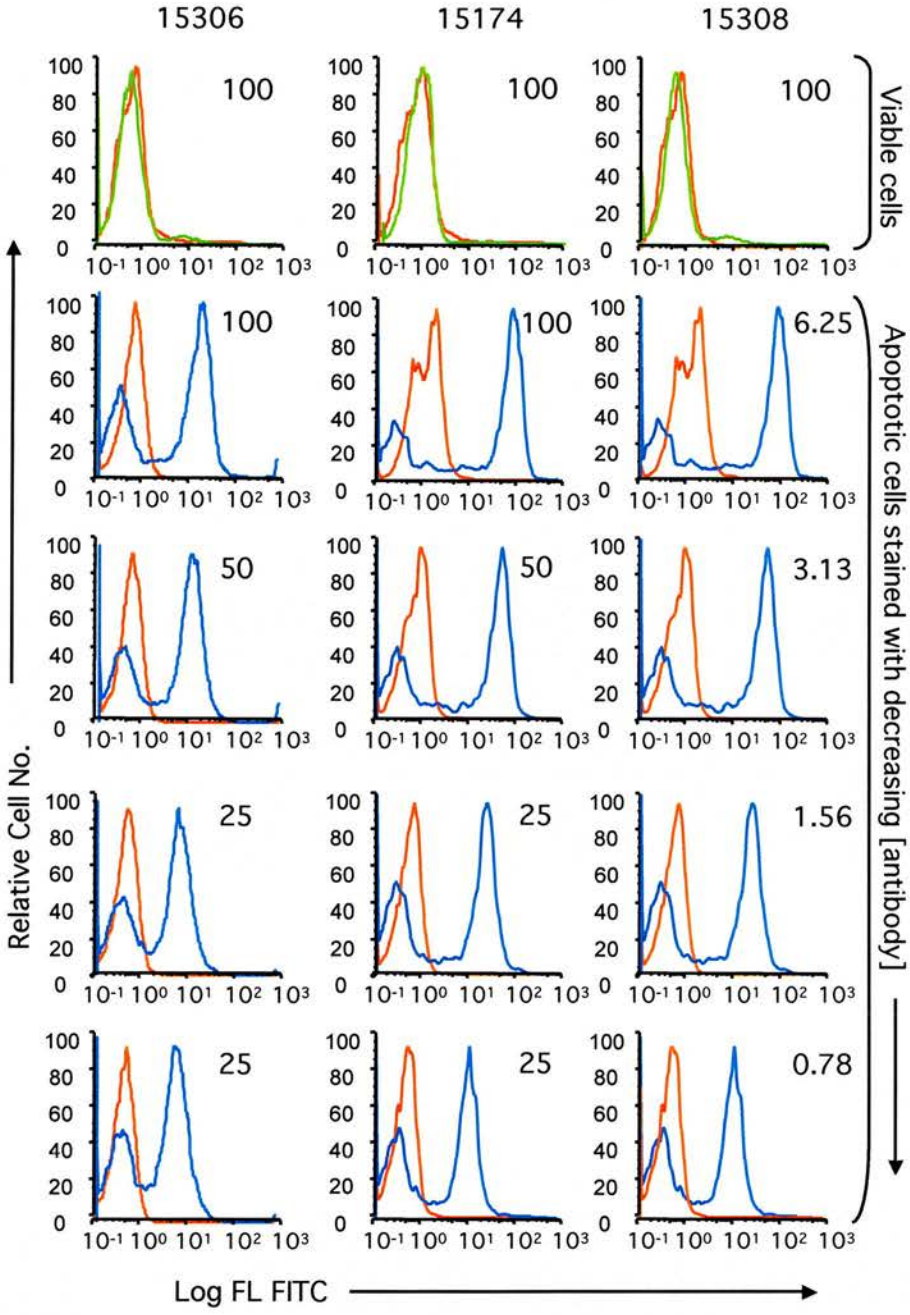


Figure 6-3. Sensitivity of anti-LPS Antibodies for Binding to Epitopes on Apoptotic Cells. Mutu I cells were induced to undergo apoptosis by treatment with ionomycin for 16h. The ability of anti-LPS Abs to bind cells was analysed by flow cytometry. Cells at a density of 2.5×10^6/ml were incubated with antibodies at the concentrations indicated to the top right of each histogram (μg/ml). After washing, bound antibody was detected with FITC labeled goat anti-mouse secondary antibody. Negative control staining with isotype-matched antibodies (γ2a for 15174 and 15306, γ3 for 15308) was performed at the same concentrations (Red histograms).
Within the population cells were defined as either apoptotic (~80%) (blue histograms) or viable (green histograms) on the basis of light scatter properties (see methods section 2.3.3.3).

6.2.1.1.1 The epitope(s) defined by mAb 15308 can be detected in permeabilised viable cells

Epitopes to which apoptotic cell-cross-reactive anti-LPS mAbs bind may be generated as a result of apoptosis or may be present, but inaccessible within viable cells due to an intact plasma membrane. Tests were therefore carried out that included a membrane permeabilisation step. Strong, uniform binding was observed when permeabilisation was performed before the addition of mAb 15308 (Figure 6-4). Binding was lost with mAbs 15306 and 15174 on apoptotic and viable cells following permeabilisation, indicating that the epitope(s) recognised are either removed or sensitive to the fixation or permeabilisation reagents used for the studies (see also section 6.2.1.3). The staining of both the apoptotic and permeabilised viable cells by mAb 15308 was further examined by confocal microscopy. Figure 6-5 shows that this antibody stains cytoplasmic regions of permeabilised viable Mutu cells and apoptotic cells. In view of the fact that mAb 15308 displayed the strongest signal when used to stain cells (figure 6-3) and that its epitope is resistant to the fixation and permeabilisation regents used for the study mentioned above, focus was mainly placed on the characterisation of this epitope in preference to that of mAb 15306 or 15174.

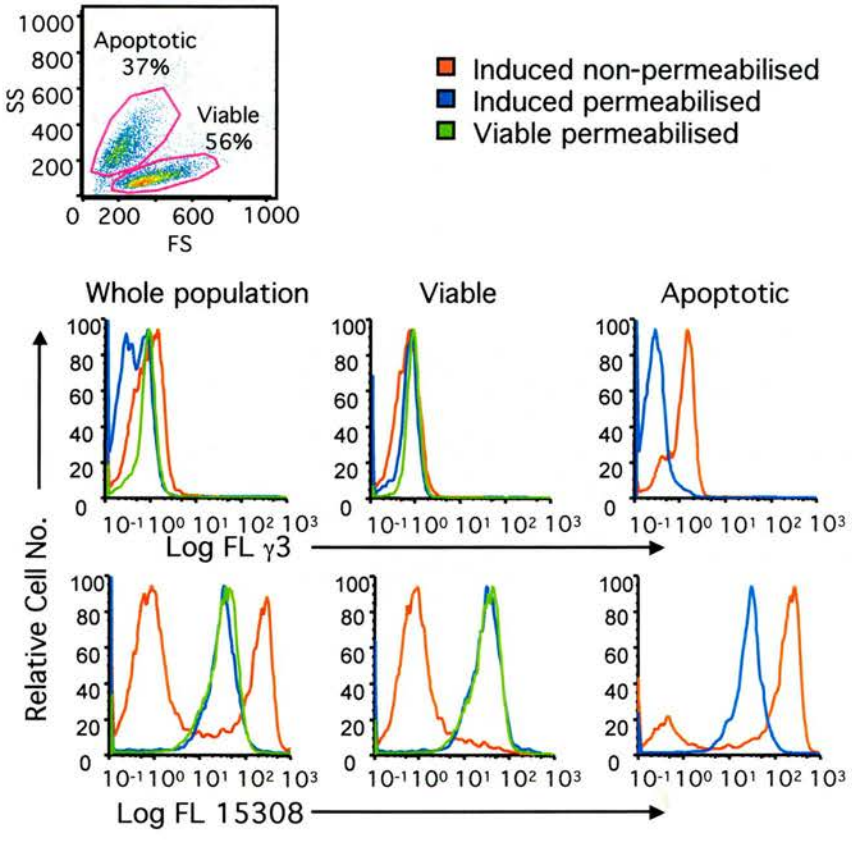


Figure 6-4. Equivalent levels of the epitope recognised by mAb 15308 can be found in both permeabilised apoptotic and viable lymphoma cells. Mutu I were treated with ionomycin for 16 hours (induced) or left untreated (Viable). Cells were then either fixed and permeabilised (using Intrastain™, DAKO) or left intact. The ability of antibody 15308 to bind cells was analysed by flow cytometry. Staining with an isotype-matched mouse monoclonal antibody (γ3) was performed as a negative control. Within the population cells were defined as either viable or apoptotic on the basis of light scatter properties (dot-plot shown for apoptosis induced population, prior to permeabilisation).
A decrease in fluorescence was observed for both 15308 and isotype-control antibody as a result of the permeabilisation process (blue vs red histogram, apoptotic cells).

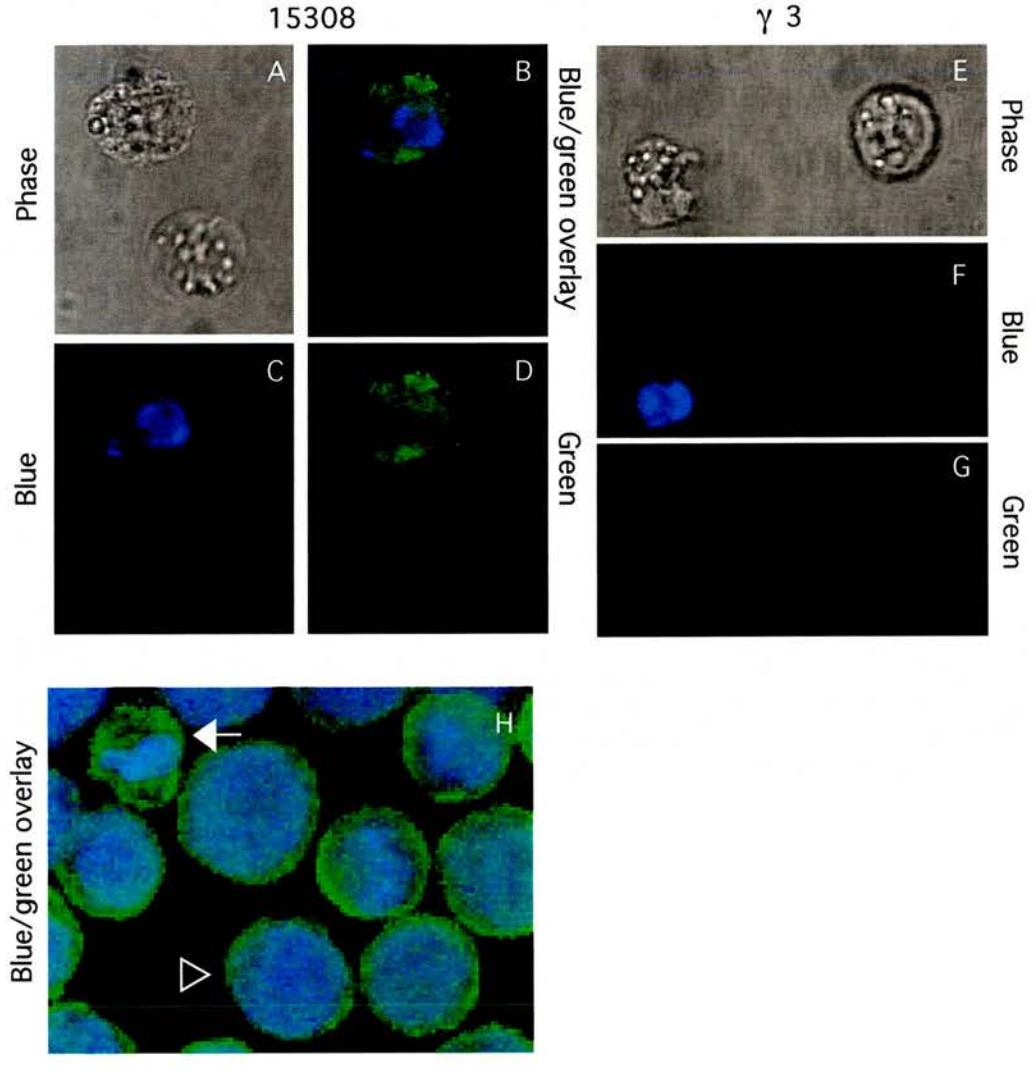


Figure 6-5. MAb15308 antibody binds an intracellular-epitope found within both viable and apoptotic cells, as determined by confocal microscopy.

Mutu I cells were treated with ionomycin for 16 hours to induce apoptosis (A-G). Binding of the anti-LPS antibody, 15308 was detected with goat anti-mouse secondary antibody labeled with alexaFluor-488 (green, B and D).

Cells were counterstained with the cell-impermeant dye, TO-PRO-3, for visualisation of nucleic acid and as a measure the integrity of the membrane (blue, B, C, F and H).

Unfixed, non-permeablised viable cells were not stained at all (lower cell, A); the isotype-matched control antibody (γ3) exhibited no staining (F and G).

When fixed and permeabilised (Intrastain ™, DAKO), Mutu I cells also exhibit cytoplasmic staining with mAb 15308 (H). Viable cells display a large rounded nucleus (open arrow head) where nuclei of apoptotic cells are condensed (closed arrow head).

6.2.1.1.2 The epitope(s) defined by mAb 15308 can be found within numerous cell types.

It was of interest to know whether the molecule(s) bearing epitopes recognised by mAb 15308 can be found in cells originating from other species and tissues, as a wide pattern of expression would be an expected feature of true apoptotic cell-associated ligands for CD14 or other pattern recognition receptors (Devitt, Pierce et al. 2003). Figure 6-6 shows examples of flow cytometry histograms assessing staining performed on primary cells (PMNs) and mouse thymoma cells (A1.1) demonstrating reactivity to cell types other than the human tumour cell line Mutu I. Indeed expression has been detected for all cell types and lineages examined (listed in table 6-3).

6.2.1.2 Localisation of epitopes defined by anti-LPS mAbs within viable adherent cells.

6.2.1.2.1 The epitope defined by mAb 15308 co-localises with microtubules

To gain further insight into the nature of the molecule(s) holding the epitope(s) recognised by mAb 15308 subcellular localisation was investigated further. Attempts to look for colocalisation with markers of known cytoplasmic organelles and cytoskeletal markers in apoptotic Mutu I cells were inconclusive due to the breakdown of cellular structures during cell death (data not shown). Given that this antibody was also seen to bind permeabilised Mutu I cells, (c.f. figure 6-4) the subcellular localisation was examined in similarly treated viable adherent cells which

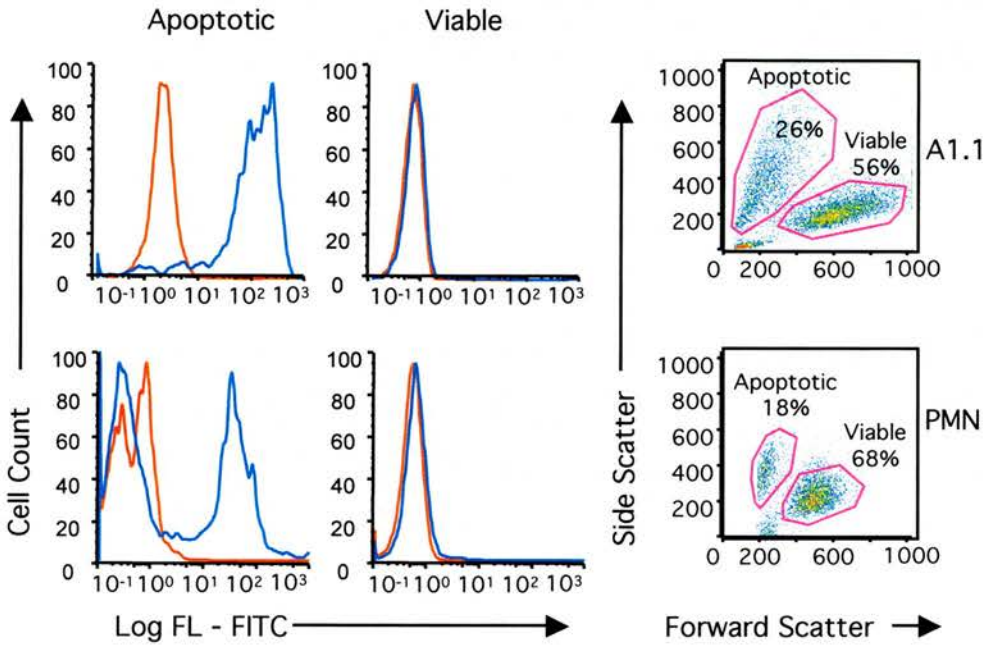


Figure 6-6. The epitope recognised by anti-LPS antibody 15308 can be found associated with apoptotic cells originating from different species and tissue lineage.

Expression of the epitope recognised by mAb 15308 was analysed by flow cytometry for populations of both primary human cells (PMN) and a mouse thymoma (A1.1) cell line. Cells were allowed to undergo spontaneous apoptosis (see methods section 2.3.3). Staining with an isotype control mouse monoclonal antibody was performed at the same concentrations as a negative control (red histograms). Bound antibody was detected with FITC labeled goat anti-mouse secondary antibody.

are more abundant in cytoplasm. Figure 6-7 shows the adherent cell line A549 prepared and stained in this way.

The "filamentous" appearance of the epitopes recognised by mAb15308 prompted investigation of colocalisation with components of the cytoskeleton. To this end, A549 cells were double-stained using anti-cytokeratin-20, anti-β-tubulin antibodies and phalloidin in addition to mAb 15308. Figure 6-7 demonstrates mAb 15308 staining appeared to colocalise with microtubules but displayed a distinct pattern of staining to cytokeratin-20 and did not colocalise with actin stress fibres. The same pattern of distribution was observed in all adherent cell lines (Figure 6-8).

6.2.1.3 Different mAbs to LPS display distinct patterns of staining within viable adherent cells.

Contrary to the observed loss of binding to Mutu I cells by mAbs 15306 and 15174 following use of the permeabilisation and fixation reagents used for this suspension cell line, staining with mAb 15174 was observed when using the most optimal permeabilisation and fixation reagents and protocol for adherent cells. A diffuse staining of cytoplasmic regions was observed with mAb 15174 (figure 6-9 panel A) compared to the filamentous localisation seen with mAb 15308 (figure 6-9 panel B) suggesting that these two α-LPS antibodies recognise different epitopes within eukaryotic cells.

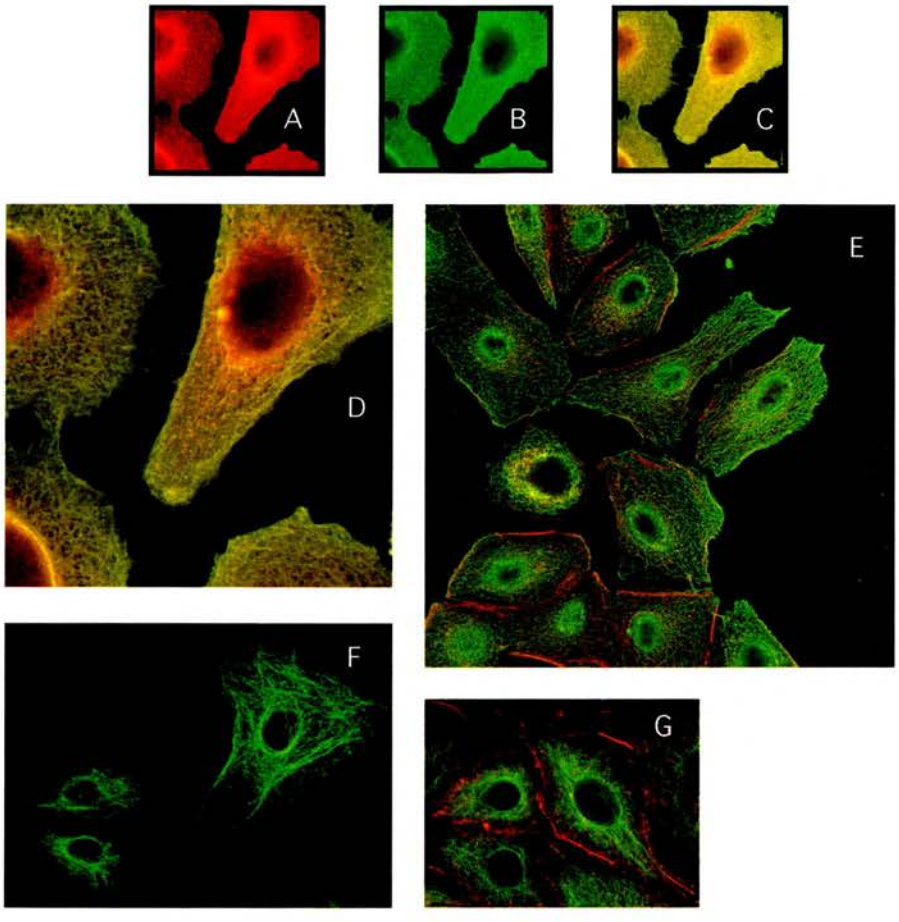


Figure 6-7. The binding of anti-LPS antibody, 15308 localises specifically with microtubules in adherent cell lines.
The human lung-epithelial cell line, A549 was grown on coverslips, fixed with 3% paraformaldehyde and permeabilised with 0.2% Triton X-100 in preparation for intracellular immunofluorescence. A-D; Cells were double-labeled with 15308 antibody and tubulin antibody. Microtubule distribution was detected by anti-β-tubulin antibody followed by alexaFluor-568 labeled secondary antibody (red, A). The distribution of 15308 antibody was visualised by secondary staining with AlexaFluor-488 labeled secondary antibody (green, B). The microtublue and 15308 images were superimposed for assessment of colocalisation (C and close up D).
On separate occasions, cells were either double-labeled with mAb15308 antibody (green) and phalloidin conjugated to AlexaFluor-568 (red) to show a distinct localisation from actin filaments (E) or single/double labeled with phalloidin conjugated to AlexaFluor-568 (red) and/or anti-cytokeratin-20 antibody (green) to visualise the distinct pattern seen with cytokeratin filaments (F)/(G).

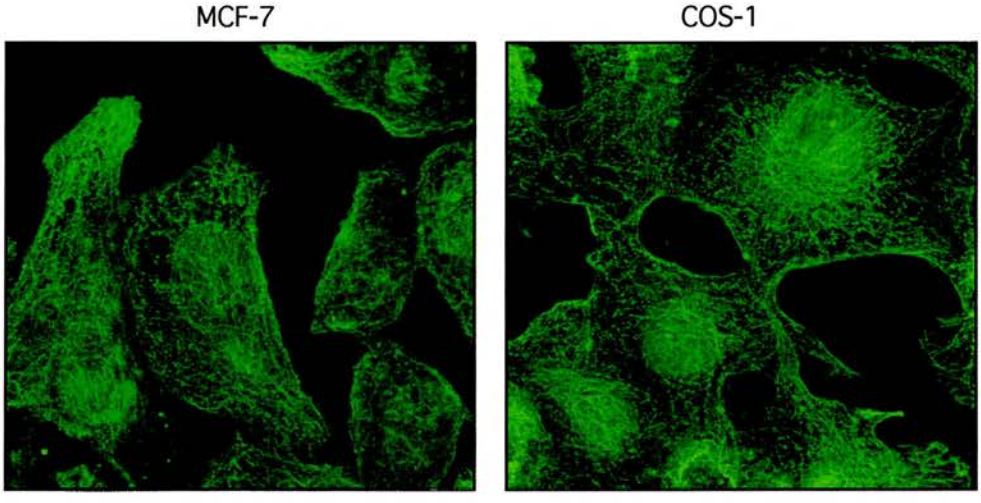


Figure 6-8. A filamentous pattern of staining with mAb 15308 is seen in numerous adherent cell lines.
The monkey kidney cell line (COS-1) and human breast adenocarcinoma cell line (MCF-7) were grown on coverslips fixed with 3% paraformaldehyde and permeabilised with 0.2% Triton X-100 in preparation for intracellular immunofluorescence. The distribution of 15308 antibody was visualised by secondary staining with AlexaFluor-488 labeled secondary antibody (green).

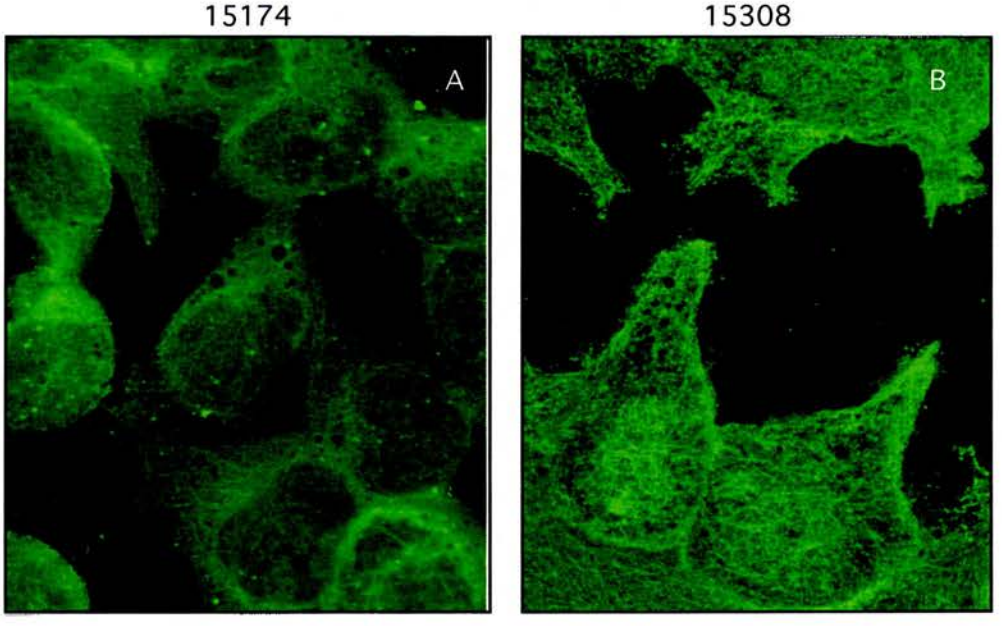


Figure 6-9. Anti-LPS mAbs 15308 and 15174 display a distinct distribution of cytoplasmic staining in adherent cell lines.
293T were grown on coverslips fixed with 3% paraformaldehyde and permeabilised with 0.2% Triton X-100 in preparation for intracellular immunofluorescence. Cells were stained with mAb 15174 (A) and mAb 15308 (B) and the distribution of bound antibody visualised by secondary staining with AlexaFluor-488 (green) conjugated secondary antibody.

6.2.2 Investigating the involvement of intracellular epitopes in the recognition of apoptotic cells by macrophages

It has been shown that for assays performed *in vitro*, features in addition to those responsible for the morphology of apoptosis (e.g. PS exposure and nuclear condensation) are required for the effective clearance of cell corpses (Devitt, Pierce et al. 2003). Furthermore, these studies suggested that apoptotic cells are most efficiently recognised at stages during programmed cell death that coincide with loss of membrane integrity.

As can be seen in figure 6-10, assessment of membrane integrity (by the exclusion of DAPI) revealed that only the feed cells with compromised membranes demonstrably bound to the surface of macrophages during a standard interaction assay. Considering that the epitopes recognised by anti-LPS mAbs (and hence potential apoptotic-cell-associated ligands of 'pattern-recognition-receptors' such as CD14) appear to be intracellular-derived epitopes, the possibility that internal structures can be recognised and accessed by macrophages was explored.

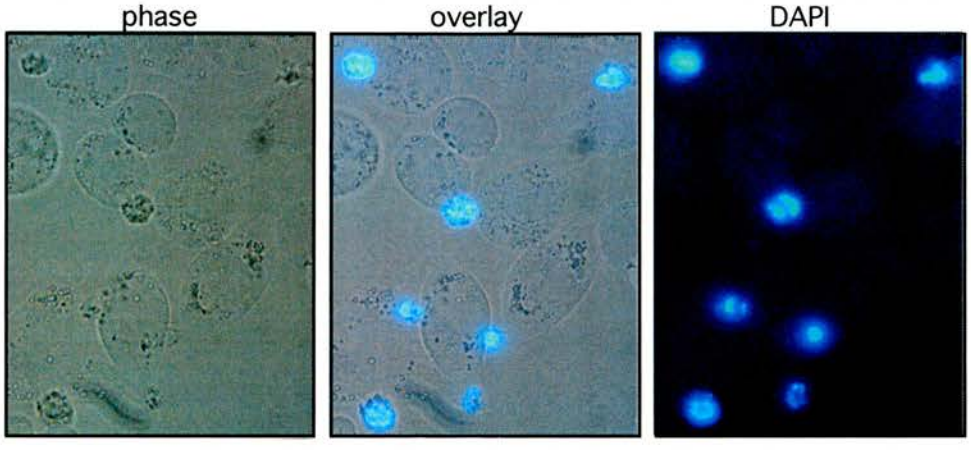


Figure 6-10. Analysis of apoptotic-feed cell membrane integrity following a macrophage interaction assay as determined by microscopy.
Mutu I cells were treated with ionomycin for 16 hours to undergo apoptosis then subjected to an interaction assay as described in section 2.10. Subsequent to co-incubation, cells were washed and membrane integrity assessed by adding the membrane impermeant dye, DAPI (blue) for 10 seconds before washing and viewing immediately by microscopy. Only those cells with comprised membrane remained firmly bound to THP-1-derived macrophages following co-incubation. Representative of three independent assays.

6.2.2.1 The recognition of apoptotic Mutu I cells by stimulated THP-1 macrophages correlates with the loss of membrane integrity.

To investigate whether ostensibly internal epitopes of apoptotic cells can serve as ligands for binding or phagocytosis, experiments were performed using Mutu I cells at a fixed period following UV-B irradiation that had been subjected to varying amounts of mechanical stress (by pipetting) to provoke accessibility of internal antigens through loss of membrane integrity. These were used in a phagocyte interaction assay with stimulated THP-1 cells representing macrophages.

(Figure 6-11 panel A). A level of pipetting sufficient to cause a loss in membrane integrity increased interaction. A good correlation between interaction of apoptotic cells and leakiness (as assessed by the uptake of PI) was seen when using feed cells at a fixed time following induction into apoptosis on several different occasions (n = 10, P value 0.0014 figure 6-11 panel B). At this point it is worth noting that permeability of cell membranes to PI may not reflect permeability to larger macromolecules. Nevertheless, increased interaction was not due to the stimulation of macrophages by a soluble factor released from the mechanically stressed cells as the incubation of non-stressed apoptotic cells and macrophages with the supernatant obtained from stressed cells did not lead to a level of interaction seen with stressed cells (figure 6-11 panel C).

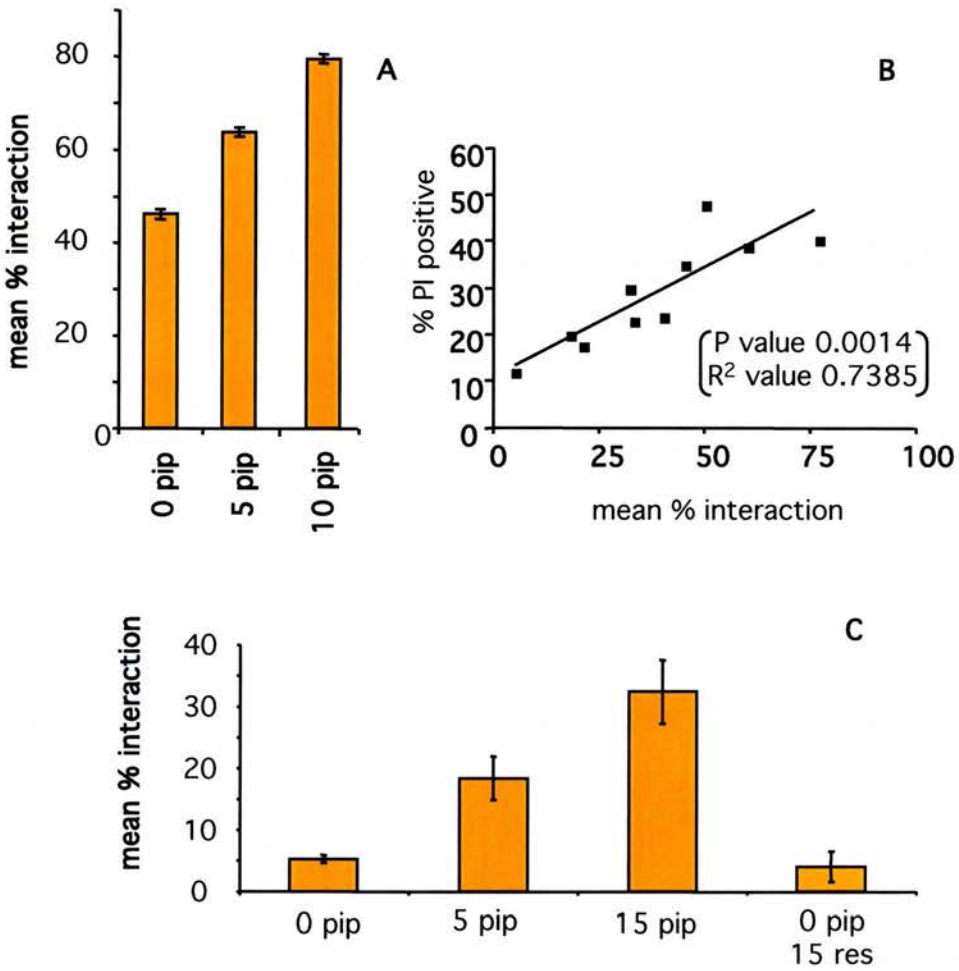


Figure 6-11. Recognition of apoptotic Mutu I cells by THP-1 macrophages correlates positively with loss of membrane integrity.
Mutu I cells were exposed to100mJ/cm^2 UV-B and left to incubate for 16 hours before use in a standard interaction assay as described section 2.10.
(A) Aliquots of resuspended cells were subjected to mechanical stress by pipetting 5 or 10 times (5 pip or 10 pip) or left untreated (0 pip).
(B) Interaction of apoptotic Mutu cells with stimulated THP-1 as a function of membrane permeability as assessed by uptake of propidium iodide (PI) ,each point represents the mean of duplicate wells.
(C) On this occasion the supernatant from cells pipetted 15 times (15 pip) was used for incubating a sample of untreated apoptotic cells during the course of the assay (0 pip 15 res). Each data point represents the mean of duplicate wells. Error bars are ± S.D. the mean values. Figures A and C are representative of 3 independent assays.

6.2.2.2 The forces required to prepare apoptotic cells for macrophage interaction assays can result in an increased rate of membrane deterioration compared to resting apoptotic cells.

Contrary to the findings of Devitt, Pierce et al. 2003, the results of some *in vitro* studies have led to the proposal that even cell populations containing very early apoptotic cells are phagocytosed by macrophages (Kurosaka, Takahashi et al. 2003). However such studies often allow long incubation periods for interaction (e.g. 4 hours) and a large excess of feed cells (which may contain a small fraction of cells at relatively late stages of death). To investigate whether these observations can be explained in other terms the rate of membrane decomposition was monitored following treatments akin to those used for preparing cell cultures for interaction assays. Figure 6-12 displays the percentage of leaky cells within apoptosis-induced cultures over a period of 4 hours that had either been left undisturbed, gently centrifuged (to simulate preparation for an interaction assay), or gently centrifuged and subjected to varying degrees of mechanical stress. A more rapid rate of membrane weakening was seen even after gentle centrifugation compared to resting apoptotic cells whereas populations subjected to vigorous pipetting in addition to centrifugation displayed an even greater rate of increase in leakiness.

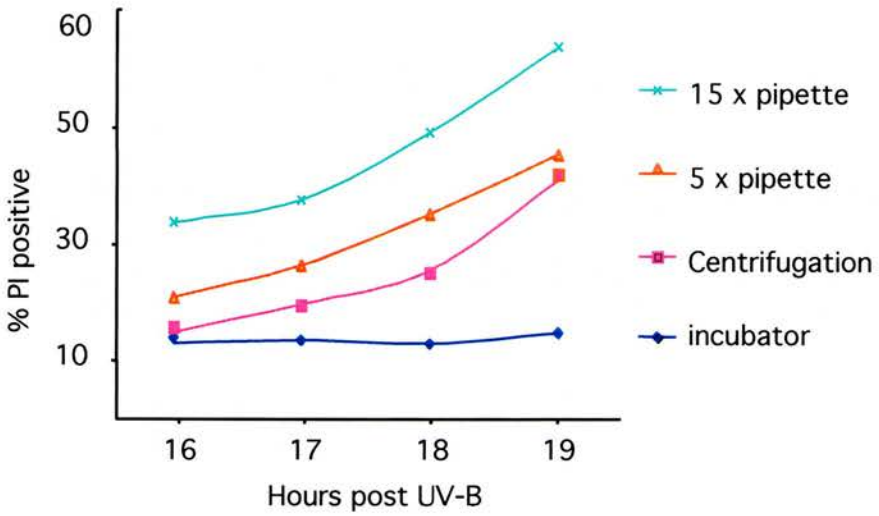


Figure 6-12. Analysis of apoptotic Mutu I cell viability in populations subjected to different mechanical treatments.
Mutu I cells were exposed to 100mJ/cm^2 UV-B and left to incubate for 16 hours before either gentle centrifugation (225g, 4 min), centrifugation plus pipetting 5 or 15 times (5 pip or 15 pip), or leaving untreated (incubator) and assessing viability by measurement of uptake of propidium iodide (PI) at hourly intervals following incubation at 37°C. On average the fluorescence of 5,000 cells was assessed by flow cytometry for each data point. Representative of 3 similar assays.

6.2.3 Involvement of epitopes recognised by anti-LPS mAbs in the recognition of apoptotic Mutu I cells by HMDM

Given that macrophage interaction appears to correlate with leakiness (or other temporally associated events) of the feed cells, and that membrane decay may occur during the course of an assay involving apoptotic cells, the possibility that recognition of apoptotic Mutu I cells by macrophages can occur through the epitopes recognised by α-LPS Abs was tested in a system that shows some CD14-dependence. Recognition was not seen to be inhibited by the presence of α-LPS Abs during the assay compared to control IgGs (Figure 6-13) despite the marked inhibitory activity of the CD14 antibody, 61D3. The same was true for assays performed using stimulated THP-1 cells (data not shown). However, given the possibility that CD14 (and other pattern-recognition receptors) can recognise many different patterns within or on an apoptotic cell, blocking a minority low-affinity ligands may be compensated by the large polyvalency of other ligands within the confined space of a phagocytic synapse. Also, it could be that the epitopes of molecules recognised by the antibodies are not the same molecular regions bound by CD14. Nevertheless, the appearance of the epitope(s) recognised by mAb 15308 and macrophage clearance are very similar.

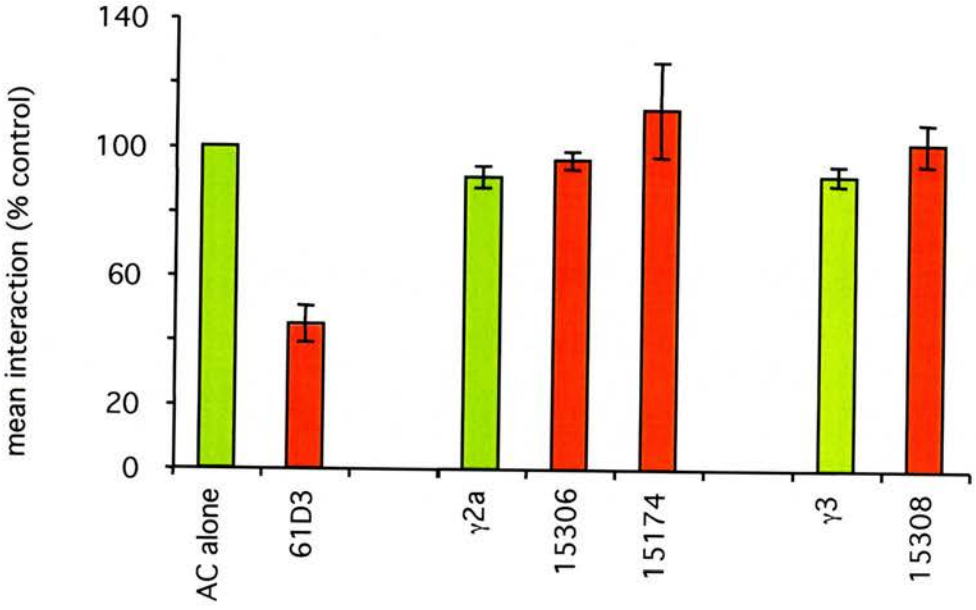


Figure 6-13. mAb blocking studies fail to provide evidence for involvement of "LPS-like" epitopes in the interaction of apoptotic Mutu cells with HMDM's. Mutu I cells were induced into apoptosis by treatment with ionomycin for 16 hours. After ionomycin treatment, Mutu cells were incubated with or without 50 μg/ml of α-LPS mouse monoclonal antibody (15306, 15174, 15308) or control antibodies (mouse IgG2a or IgG3) in assay medium (RPMI 1640-HEPES containing 0.2% BSA) at 0 °C for 30 min, washed, and subjected to the binding assay. All the data are the means ± S.E.M. of triplicate experiments using macrophages derived from different donors.

6.2.4 MAb 15308 binds to a Western blot of Mutu I cell lysates separated by SDS-PAGE

Having found that anti-LPS antibodies can associate with apoptotic cells, it was of interest to investigate the structural basis of this interaction in terms of the molecular species that form these "LPS-like epitopes". Whilst many methodologies could be applied in an attempt to gain such insights (see general discussion section 8), the initial approach taken towards identification of the species recognised by these antibodies was to assess preferential binding of α–LPS Abs towards Mutu I cell lysates separated by SDS-PAGE by Western blot analysis (Figure 6-14). Notably, one band with an approximate molecular mass of ~40 kDa present in the lysates of cells was detected by mAb 15308. No band with this mobility was seen for the other anti-LPS mAbs or control antibodies.

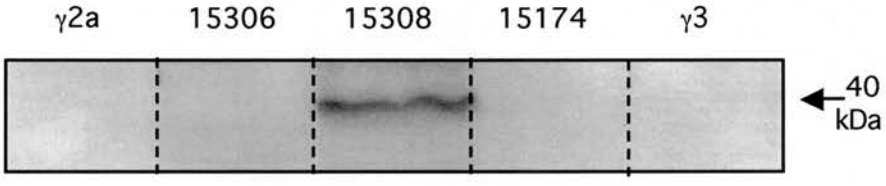


Figure 6-14. Screening of Mutu I cell lysates with anti-LPS antibodies.
A lysate of Mutu I cells was prepared and separated by electrophoresis through a 10% polyacrylamide SDS gel before transferring to PVDF membrane. The resultant membrane was cut into strips and probed with the antibodies indicated above the blot. Isotype-matched antibodies were γ2a (for 15306 and 15174) and γ3 (for 15308).

6.3 Summary

The antibody staining studies described in this chapter suggest that: -

- Viable eukaryotic cells hold intracellular epitopes that are also associated with microbial organisms.
- These epitopes can become exposed during apoptosis.

In addition, through assessing the ability of apoptosis-induced cells, destined for use in macrophage interaction assays, to take up vital dyes, it was possible to demonstrate that: -

- Following a standard macrophage interaction assay the only apoptotic Mutu I feed cells that remain firmly bound to macrophages are those with a compromised membrane integrity.
- Increasing the permeability of apoptotic cells by mechanical disruption leads to an increase in the ability of macrophages to interact with apoptotic cells.
- The preparation of cells that have been induced into apoptosis for macrophage interaction assays by standard procedures leads to an increased rate of membrane breakdown.

Thus, in this system, the onset of engulfment appears to correlate with the loss of membrane integrity. These findings advocate the possibility that apoptotic cells present internal epitopes to macrophages engaging in apoptotic-cell clearance. Considering that the appearance of epitopes recognised by antibodies to LPS display similar kinetics, there is an opportunity for these molecules to participate in

engulfment. However, blocking of apoptotic-cell recognition by HMDM or stimulated THP-1 by these antibodies was not observed.

Finally, anti-LPS mAb 15308 showed activity towards an ~40 kDa molecule from Mutu I whole cell lysates by Western blot analysis. To ascertain whether this molecule participates in apoptotic-cell recognition, focus was placed on its identification and characterisation, the results of which are presented in the following Results chapter.

7 Results Chapter 4: Characterisation of the Apoptotic-Cell-Associated Epitope Recognised by anti-LPS Monoclonal Antibody 15308.

7.1 Introduction

The data presented in chapter 3 established that antibodies previously defined as being specific for LPS can be observed to bind to late apoptotic cells. One of the cross-reactive mAbs, 15308, bound to a ~40 kDa band from lysates of Mutu I BL cells on a denaturing gel.

The work described in this chapter identifies the ~40kDa molecule which bears an epitope recognised by mAb 15308 as the 37/67kDa Laminin Receptor (laminin binding protein, (LBP/p40). Confirmation of binding to LBP/p40 exogenously expressed in both mammalian and bacterial systems will be explored and attempts made to purify recombinant LBP/p40 for functional studies with relevance to CD14-dependent apoptotic cell clearance by macrophages. In addition, evidence for the appearance of the epitope defined by mAb 15308 on the surface of apoptotic cells prior to loss of membrane integrity will be presented.

7.2 Results

7.2.1 Identification of mAb 15308-reactive species present in Mutu I cells

7.2.1.1 15308 binding to sub-cellular fractions

In section 6.2.4, mAb 15308 was seen to bind to a species present in lysates of Mutu I BL cells on a denaturing gel that resolves at ~40kDa. Within cell lysates, many molecules are likely to resolve at a similar position to this, making identification of the target species difficult. Considering that mAb 15308 has been shown to locate to definite regions within cells (for example microtubules - section 6.2.1.2.), attempts were made to reduce the level of non-reactive background molecules in the cell lysates being studied by exploring reactivity of the antibody towards various sub-cellular fractions, with the intention of using mAb 15308-reactive fractions for identification of the target species by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Figure 7-1 illustrates the relative reactivity of mAb 15308 associated with cellular fractions isolated from Mutu I cells by differential centrifugation. SDS/PAGE and western blot analysis of 15308 reactivity revealed major differences amongst the four fractions, with the majority of the reactivity (~40kDa) found within fraction P1 (which corresponds to large organelles and protein complexes). Less was observed in fraction P2 (which corresponds to insoluble membrane fraction). A faint reactivity of higher molecular weight (~65kDa) was seen in the final soluble fraction. The significance of the reactivity in the last fraction will be discussed further in section 7.2.1.3).

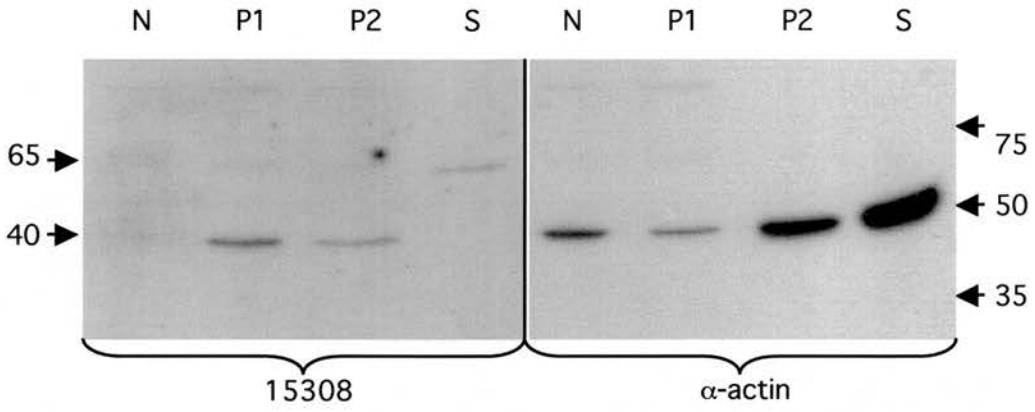


Figure 7-1. SDS-PAGE and Western Blot analysis of Mutu I cell lysate fractions.
A lysate of Mutu I cells was prepared and fractionated by sequential centrifugation into a 1,000g pellet (N), 27,000g pellet (P1) 100,000g pellet (P2) and remaining supernatant (S). Pellets were resuspended in a volume of lysis buffer equal to that remaining in the final supernatant. Equal volumes of the fractions were electrophoresed in the positions as indicated above the lanes through a 10% polyacrylamide SDS gel and transferred to PVDF membrane before blotting with antibodies indicated below the blots.

7.2.1.2 Protein identification by matrix-assisted laser desorption–ionization/ time-of-flight-mass spectrometry (MALDI-TOF-MS) analysis

Genome sequence information in combination with data produced by matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) mass spectrometers has provided a convenient and powerful resource for protein identification. Database searching allows identification of proteins using the data produced by MALDI/TOF spectrometry.

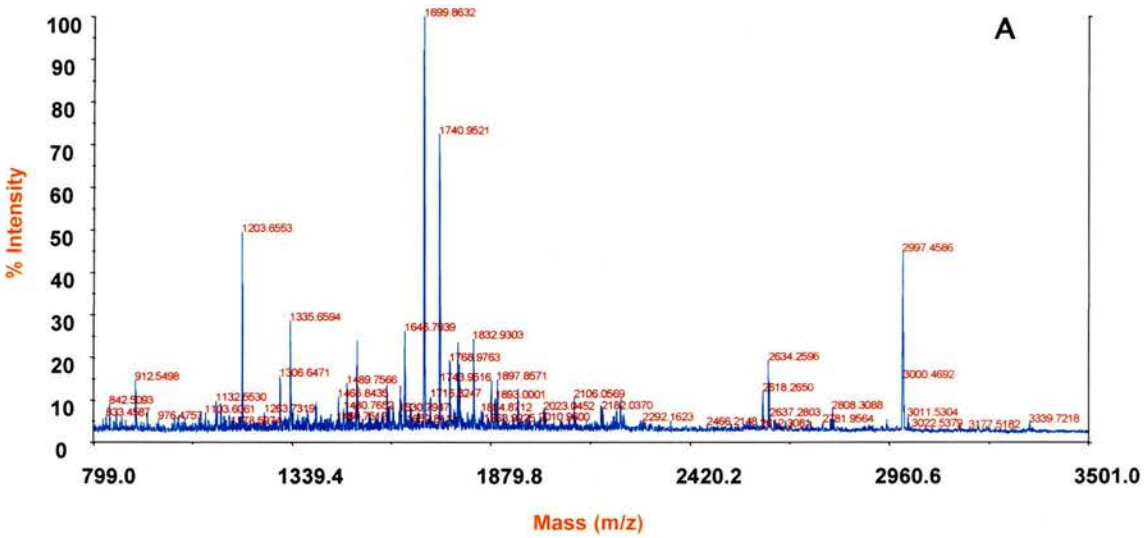
These approaches were applied to the identification of the mAb 15308-reactive band present in the electrophoretically-separated protein mixtures of Mutu I cells. Thus, larger scale Mutu I cell lysates were prepared and fractionated (see methods section 2.2.3) and two identical gels were used to separate fraction P1 of lysed cells. Following separation, one gel was stained for total protein content and the other Western blotted and probed with mAb 15308. Comparison of blot and gel suggested that the mAb 15308 reactive band might be associated with any of three discrete bands of similar size.

The three bands were excised and peptides contained within tryptic digests of them analysed by MALDI-TOF-mass-spectrometry. The suggested identities of the three species were: β-Actin, Laminin binding protein (LBP/p40) and a Septin named NEDD5 (neuronaly expressed, developmentally downregulated-5).

β-Actin was not considered a candidate mAb 15308-reactive protein as distinct patterns of localisation were seen when co-staining with phalloidin (see section 6.2.1.2). Moreover, Western-blot analysis of fractionated cell lysates had shown

actin to be present in different relative proportions to the molecule bearing the 15308 epitope (figure 7-1).

At this point it is worth noting that in addition to identifying the mAb 15308-reactive species by direct analysis of cell lysate fractions, immuno-precipitations with mAb 15308 were attempted. However in gel visualisation of SDS-PAGE-separated protein bands obtained from the precipitation revealed the presence of only a ~25 kDa species when compared to the control immuno-precipitation with an isotype-matched antibody. MALDI-TOF-MS analysis identified this band as 40S ribosomal protein, S3A. This protein is of lower molecular weight than that recognised by mAb 15308 so was not considered as a candidate for direct recognition by the antibody. However, the possibility that it co-immunoprecipitates with the true mAb 15308 reactive species is discussed in section 7.2.1.3. Table 7-1 and figure 7-2 show summaries of the MALDI-TOF-MS analysis.



KEEDVLKFLAAGTHLGGTNLDFQMEQYIYKRKSDGIYIINLKRT B
WEKLLVAARAIVAIENPADVSVISSRNTGQRAVLKFAAATGA
TPIAGRFTPGTFTNQIQAAFREPRLLVVTDPRADHQPLTEASY
VNLPTIALCNTDSPLRYVDIAIPCNNKGAHSVGLMWWMLAR
EVLRMRGTISREHPWEVMPDLYFYRDPEEIEKEEQAAAEKAVT
KEEFQGEWTAPAPEFTATQPEVADWSEGVQVPSVPIQQFPTE
DWSAQPATEDWSAAPTAQATEWVGATTDWS

Figure 7-2. Matrix-assisted Laser Desorption and Ionization-Time of Flight (MALDI-TOF) Mass Spectrometric Analysis of the putative protein recognised by mAb 15308.

Polypeptide bands of interest were excised from a 10% polyacrylamide SDS gel and digested in trypsin. Tryptic peptides were extracted from the gel prior to MALDI-TOF mass spectrometric analysis. Resultant peptide masses were used to search the SwissProt and MASCOT databases. A: the MALDI-TOF mass spectrum for the tryptic digest of the peptide band identified as Laminin-Binding Protein. B: summary of the output from the database query. The matched peptides covered 58% of the total protein as indicated in red font (168/285 amino acids)(MOWSE score, 2.330e+10).

Identity	Predicted Mass (kDa)	%Coverage	Accession No.
Beta-actin	40.2	48	AAH08633
Laminin-binding protein	31.8	58	X61156.1
NEDD5	41.5	39	BC014455.1
40S ribosomal protein S3a	29.9	42	NM_001006

Table 7-1. Summary of matrix-assisted laser desorption-ionization/time-of flight (MALDI-TOF) mass spectrometry analysis of peptide mixtures resulting from the tryptic digestion of candidate proteins. Predicted mass; molecular weights calculated from primary amino-acid sequences. % Coverage; percentage of total protein which matched peptides covered. Accession No.; gene identity.

7.2.1.3 Consideration of LBP/p40 as a candidate molecule bearing an epitope recognised by mAb 15308.

The 37kDa precursor of laminin-binding protein (LBP/p40) also known as 32/67-kD laminin receptor (LR) (Clement, Segui-Real et al. 1990) or 37/67-kD LR (Castronovo, Claysmith et al. 1991) is an evolutionarily conserved, multifunctional protein which can be found distributed on the cell surface associated with $\alpha_6\beta_4$ integrin as laminin-binding protein in its 67kDa form (Ardini, Tagliabue et al. 1997), on 40S ribosomes as a component of the translational machinery (Rosenthal and Wordeman 1995; Ardini, Pesole et al. 1998), and to a lesser extent bound to histones in the nucleus (Sato, Kinoshita et al. 1996).

The terminology used by previous investigators of this protein often relates to the particular biological function or sub-cellular localisation studied. Discrepancies arise from the fact that cDNA clones of the 67 kDa laminin receptor only contain coding potential for a 32 kDa polypeptide (Clement, Segui-Real et al. 1990), while the translation product of the corresponding mRNA has a molecular mass of 37-40 kDa and acts as a precursor of the 67 kDa LR (discussed in more detail section 8).

LBP/p40 has been described as an abundant cytoplasmic protein expressed in numerous cell types and at elevated levels in malignancies including breast, colon, prostate, lung, liver, ovary cancer, and lymphoma (Yow, Wong et al. 1988; Rescan, Clement et al. 1991; al-Saleh, Delvenne et al. 1997; Fontanini, Vignati et al. 1997; Menard, Tagliabue et al. 1998).

Through study of tumour samples the oncogenic potential of LBP/p40 has been realised, and functional studies suggest it is involved in interactions between cancer cells and laminin during tumour invasion and metastasis (Yow, Wong et al. 1988;

Mafune and Ravikumar 1992; Starkey, Uthayakumar et al. 1999; Tanaka, Narumi et al. 2000).

Certain information presented in the literature regarding this protein is in keeping with the following features of the mAb 15308 reactivity: -

(1) Recognition of an epitope present in the cytoplasm of numerous cell types (described in results chapter 3).

(2) Distribution of reactivity towards fractionated cell lysates (fraction corresponding to sub-cellular organelles and large protein complexes section 7.2.1.1).

(3) Immuno-precipitation of components of the translational machinery (Ribosomal subunit S3a section 7.2.1.2).

Consideration of this evidence led to focusing on the possibility that LBP/p40 contains the epitope to which 15308 binds.

7.2.2 PCR amplification and molecular cloning of LBP/p40 cDNA

To generate a cDNA fragment of LBP/p40 from Mutu I total mRNA, a PCR was carried out using primers based on the complete coding sequence of the gene (accession X61156.1) cited in the records obtained from the primary result of MALDI-TOF-MS analysis (Yow, Wong et al. 1988). The same approach was also employed for NEDD-5, in the event that this candidate from MALDI-MS analysis held the epitope recognised by mAb 15308. As shown in figure 7-3, the reactions yielded single cDNA fragments of approximately 890 bp for LBP/p40, 1050 bp for NEDD5 (accession BC014455.1) (Strausberg, Feingold et al. 2002), and 528 bp for GAPDH. These were all consistent with the sizes expected for their respective mRNA transcripts.

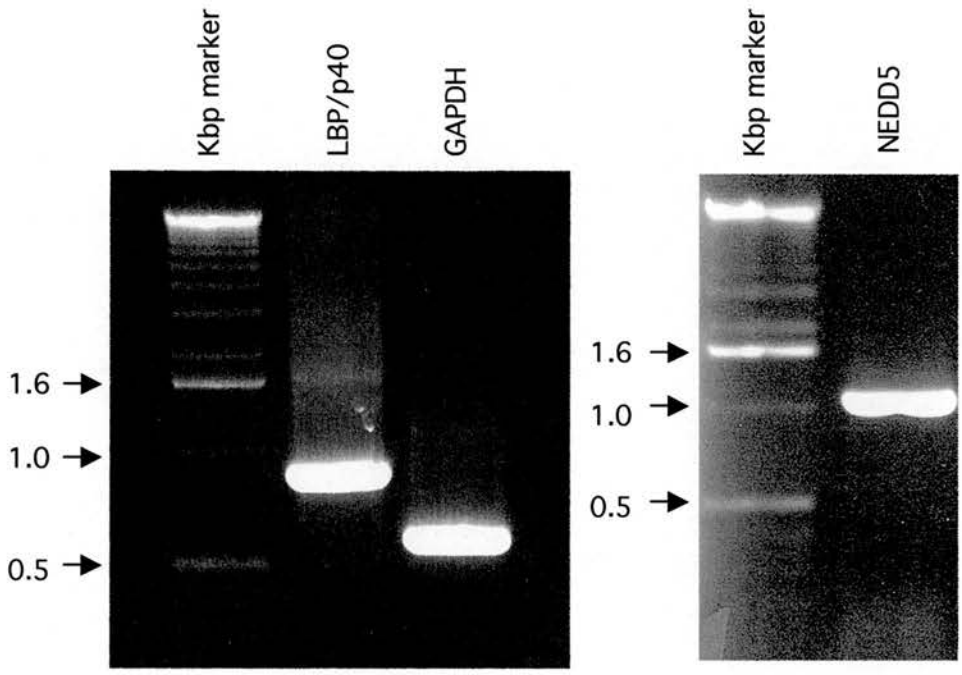


Figure 7-3. Detection of laminin-binding protein (LBP/p40) and NEDD5 in Mutu I cDNA by PCR. Primer sets pCDNA3.1D/LBP/p40-V5-His and pCDNA3.1D/NEDD5-V5-His resulted in PCR product sizes, approximately 890 and 1050bp for LBP/p40 and NEDD5 respectively. Primer sets for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a quality control for cDNA production (528 bp). Kbp; kilo-base pair.

7.2.3 Expression and detection of LBP/p40 in 293T cells and detection of by immunoblotting with mAb 15308

To determine the ability of mAb 15308 to bind exogenously expressed LBP/p40, HEK293T cells were transfected with pCDNA3.1D/LBP/p40-V5-His (see section 2.1.4 for plasmid construction). Control cells were either mock transfected without plasmid DNA or transfected with pCDNA3.1D/NEDD5-V5-His. Cell lysates were run on SDS–PAGE gels and detected by subsequent Western blotting both with an antibody to the V5 epitope (encoded by the expression vector) and with mAb 15308. Exogenously expressed LBP/p40 was observed as two species of approximately 40 kDa and 50-70 kDa when using the α-V5 antibody for detection (figure 7-4). This is consistent with both the faint band seen in fraction S of Mutu I lysate (figure 7-1) and the 67kDa form of this protein. Exogenously expressed cloned NEDD-5 was detected at ~42 kDa when using the α-V5 antibody for detection. No bands were found with this antibody in the lysates of mock-transfected cells.

MAb 15308 was seen to react with the corresponding exogenously expressed cloned LBP/p40 species but neither NEDD5 nor any bands in addition to the endogenous reactivity found in lysates of mock-transfected cells. No reactivity corresponding to cloned LBP/p40 was seen when probed with γ3 isotype-matched antibody to 15308 or an α-β-actin antibody. The differences in signal intensity between detection using α-V5 and mAb 15308 for the exogenously expressed LBP/p40 can be explained by the high affinity of α-V5 to its epitope with detection of as little as 2pg of a 50 kDa recombinant protein bearing the V5 tag being clearly detected in Western-blotted

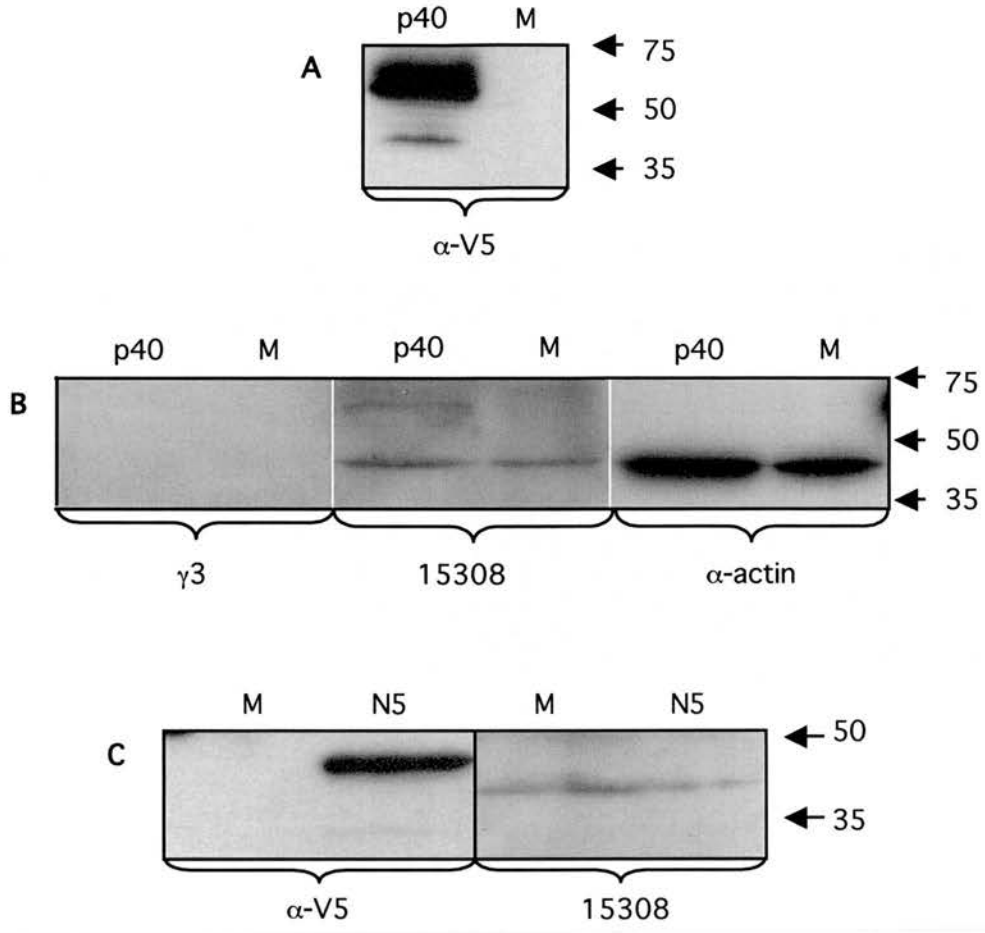


Figure 7-4. Binding of anti-LPS mAb 15308 to LBP/p40 exogenously expressed in HEK293T cells as shown by immunoblotting. HEK293T cells were transiently transfected with pCDNA3.1D/LBP/p40-V5-His or pCDNA3.1D/NEDD5-V5-His encoding the V5-tagged products LBP/p40 (p40) and NEDD5 respectively, or mock transfected (M). Cell lysates were separated by electrophoresis through a 10% polyacrylamide SDS gel and transferred to PVDF membrane before blotting with the antibodies indicated below the blots. A: Detection of the transiently expressed LBP/p40 using an antibody to the V5 tag reveals two species of approximate molecular weight 40kDa and 50-70kDa. B: Transiently expressed LBP/p40 can be detected with mAb 15308 but not with an isotype control antibody (γ3). Probing with an antibody to actin indicates the amount of whole cell-lysate loaded in each sample. C: Transiently expressed NEDD5 can be detected with α-V5 mAb but not mAb 15308 (which detects only endogenous LBP/p40).

SDS-PAGE gels (personal communication - Invitrogen Technical support). These results confirm the presence of an epitope recognised by the α-LPS 15308 in cloned LBP/p40 exogenously expressed in mammalian cells.

7.2.3.1 Expression of polyhistidine-tagged-LBP/p40 and purification by nickel metal-affinity.

In order to study further the role of LBP/p40, notably in the context of apoptotic-cell clearance and interaction of its "LPS-like" epitope (as defined by mAb 15308) with LPS-binding PRRs, specifically CD14, attempts were made to purify recombinant forms of the protein for use in functional studies. Initially, recombinant poly-histidine tagged LBP/p40 (rLBP/p40) was expressed and purified using three systems; (1) as a secreted protein from the culture supernatants of stably transfected K562 cells, (2) from lysates of *E. coli* or (3) from lysates of stably-transfected MCF-7 cells (refer to methods sections 2.1.4 to 2.1.6 for plasmid construction).

In all cases, nickel metal-affinity resin columns were used for one-step purifications of His-tagged rLBP/p40 constructs. The resultant protein-containing fractions were dialysed against PBS to remove imidazole and the purity of the protein was examined by comparing total-protein-stained SDS–polyacrylamide gels to Western blots probed with α-V5 Ab, mAb 15308 or a commercially available antibody to LBP/p40 (Lam-R) which became available for later experiments.

With these tools the intention was to test;

- The ability of LBP/p40 to bind CD14-expressing cells.
- Recombinant LBP/p40 preparations for the ability to block CD14-dependent apoptotic-cell interactions
- The relationship of the laminin-binding site of LBP/p40 to the epitope recognised by mAb15308.

7.2.3.2 Expression of LBP/p40 in *E. coli*

DNA encoding LBP/p40 was sub-cloned from pCDNA3.1D/LBP/p40-V5-His into pTrcHis2C (see section 2.1.6.2 for plasmid construction) for expression in *E. coli*. Expression in *E. coli* was confirmed by probing Western blots of pTrcHis2C-LBP/p40pm transformed and non-transformed cell culture lysates with an antibody to the V5 tag of the recombinant protein and mAb 15308 (figure 7-5 panel A). High-level expression of rLBP/p40 was induced with the addition of 1mM IPTG to cell cultures prior to purification from cell lysates following sonication. As was seen with 293T cells transiently-transfected with LBP/p40 (section 7.2.3, figure 7-4), the molecular sizes observed for the bands of the bacterially expressed rLBP/p40 species by Western blot using α-V5 were approximately 40 kDa and 50-70 kDa for figure 7-6 panel C. MAb 15308 and Lam-R antibody also detected the recombinant protein (figure 7-6, panels A and B).

Examination of total-protein-stained SDS–polyacrylamide gels (figure 7-5 panel B), revealed the presence of protein bands in addition to those of LBP/p40 species observed by probing Western blots of the recombinant protein with either α-V5, mAb 15308 or mAb Lam-R. Although indicating that the preparation was not of

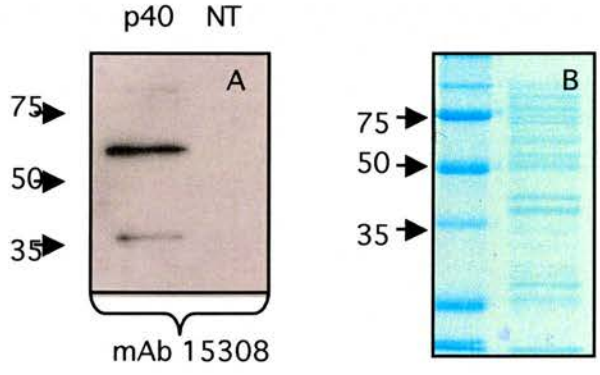


Figure 7-5. Expression of the recombinant LBP/p40 (rLBP/p40) in *E. coli*.

A; The presence of recombinant protein in rLBP/p40 (p40)-transformed or non-transformed (NT) *E. coli* cell lysates was detected by probing Western blots of total protein with mAb 15308. Equal amounts of total protein were loaded to lanes of SDS-page gels for each cell type.

B; The presence of proteins other than LBP/p40 in nickel-metal affinity-purified rLBP/p40 preparations was assessed by examination of 10% SDS-polyacrylamide gels stained for total protein.

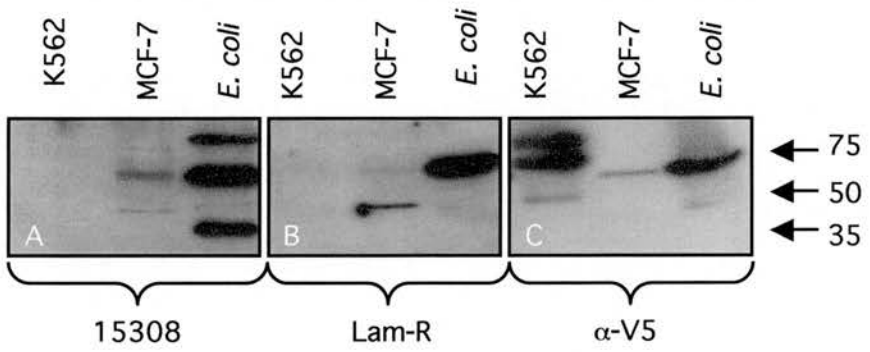


Figure 7-6. Assessment of Nickel-metal Affinity-Purified Recombinant LBP/p40 Derived From Different Expression Systems. LBP/p40 was expressed as an intracellular protein in *E. coli* and MCF-7 cells or as a secreted protein in K562 cells. Western blots of purified LBP/p40 preparations were analysed by probing with mAb 15308 (A), Lam-R Ab (B, a commercially available anti-LBP/p40 Ab) or anti-V5 (C). Equal amounts of total protein were loaded to lanes of SDS-page gels for each rLBP/p40 preparation.

high purity, the presence of these contaminating proteins in greater amounts than recombinant LBP/p40 confirms the specificity of mAb 15308 to this protein. Furthermore, reactivity towards the protein expressed in a bacterial system provides information that eukaryote-specific processing is not required for binding to LBP/p40 by mAb 15308.

7.2.3.3 Stable expression of LBP/p40 in MCF-7 cells and purification from cell-lysates.

For purification of LBP/p40 from an endotoxin-free mammalian system, MCF-7 cells were transfected with pCDNA3.1D/LBP/p40-V5-His by electroporation and selected for stable expression rLBP/p40 by resistance to neomycin. Expression was confirmed by probing Western blots from cell lysates of transfected and non-transfected cells and by probing permeabilised transfected cells with α-V5 mAb by indirect immunofluorescence (figure 7-7 panels A and B) prior to purification of the histidine-tagged recombinant protein from cell lysates. The presence of recombinant protein in purified preparations was assessed by western blot using α-V5 and mAb 15308 and mAb Lam-R (figure 7-6). As was seen for 293T cells transiently-transfected with this construct and bacterially-expressed LBP/p40 (sections 7.2.3 to 7.2.3.2), the molecular sizes observed for the bands were approximately 40 kDa and 50-70 kDa for the purified rLBP/p40 species.

Again, examination of total-protein-stained SDS–polyacrylamide gels revealed the presence of protein bands in addition to those of LBP/p40 species observed by probing Western blots (figure 7-7 panel C).

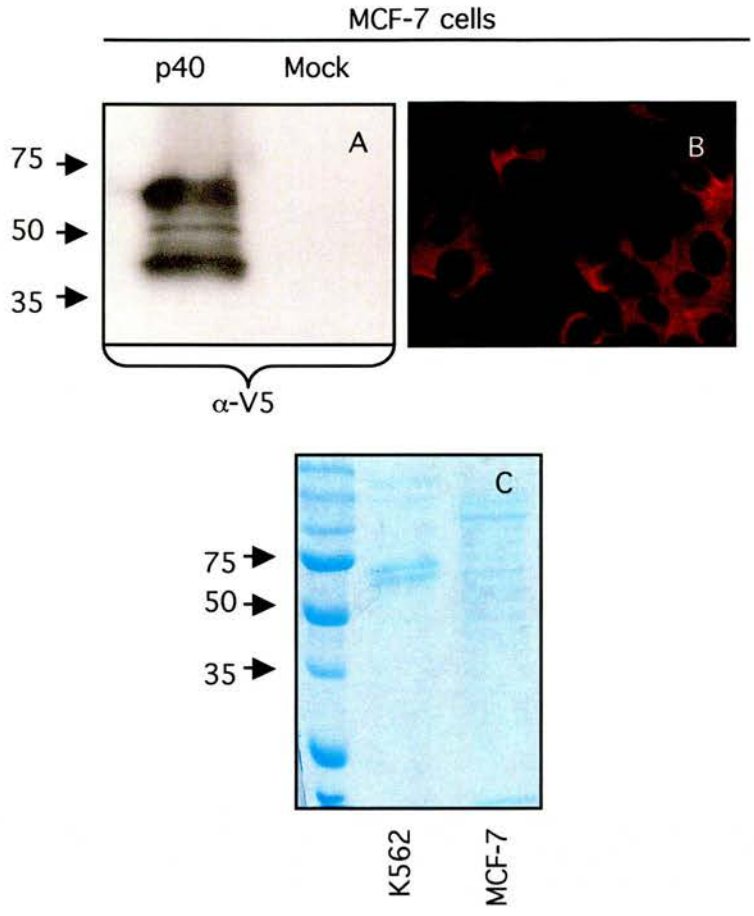


Figure 7-7. Expression of the recombinant LBP/p40 (rLBP/p40) in MCF-7 cells and K562 cells.
The presence of rLBP/p40 in MCF-7 cells was detected by probing Western blots of total protein with α-V5 mAb (panel A) or by indirect immunofluorescence using α-V5 antibody followed by AlexaFluor-568 labeled secondary antibody (red, panel B). The presence of proteins other than LBP/p40 was assessed by examination of 10% SDS-polyacrylamide gels stained for total protein (C)(a preparation obtained from expression in K562 cells is also shown for comparison).

7.2.3.4 Expression of secreted LBP/p40 in K562 cells.

DNA encoding LBP/p40 was sub-cloned from pCDNA3.1D/LBP/p40-V5-His into pSecTag2/HygroA (see methods section 2.6.1.3 for plasmid construction) to allow transport of the translated protein into the secretory pathway for release from cells. K562 cells were transfected with pSecTag2/HygroA-LBP/p40 by electroporation and a Hygromycin-resistant clone, expressing high levels of recombinant protein (Clone A2) identified by screening of culture supernatants by ELISA (figure 7-8 panel A). Cultures of Clone A2 were grown in serum-free growth medium (Sigma hybridoma medium, animal component-free) to saturation prior to purification of the histidine-tagged recombinant protein from cell-free supernatants. The presence of recombinant protein in purified preparations was assessed by Western blot using α-V5 and mAb 15308 and mAb Lam-R (Figure 7-6). Contrary to the observed molecular sizes for the V5-containing bands present in preparations derived from MCF-7 cells or *E. coli* (~40 kDa and 50-70 kDa) (sections 7.2.3.2 and 7.2.3.3), recombinant LBP/p40 as a secreted protein in K562 cells was found to be expressed as three main species of molecular weight ~50 kDa, ~75 kDa (approximately 10 kDa greater than in MCF-7 and *E. coli* seen more clearly in figure 7-8 panel B) and ~80kDa. Significantly, neither mAb 15308 nor Lam-R antibody were able to detect the recombinant LBP/p40 expressed in this way.

Comparison of the relative amounts of recombinant protein in this preparation with the MCF-7-derived preparation by ELISA, detecting the presence of a V5 epitope, revealed there to be much more V5-tagged protein in the K562-derived sample (figure 7-9).

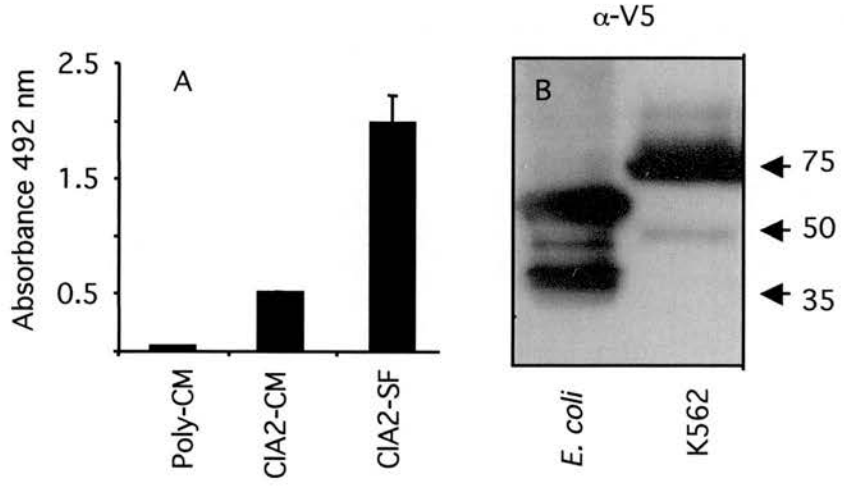


Figure 7-8. Expression of the recombinant LBP/p40 (rLBP/p40) in K562 cells.

A; Recombinant protein in supernatants of rLBP/p40 transfected K562 cells was detected in an ELISA for the V5 epitope. Poly-CM: polyclonal culture of rLBP/p40-transfected K562 cells expressing varying levels of rLBP/p40. ClA2-CM: a rLBP/p40-transfected K562 cell clone expressing high levels of recombinant protein grown in complete culture medium. ClA2-SF: a rLBP/p40-transfected K562 cell clone expressing high levels of recombinant protein grown in serum-free culture medium. The absence of serum proteins (which compete for binding sites on plastic wells) in this sample allowed for a stronger ELISA signal than for the ClA2-CM sample.

B; size difference between intracellular bacterial and secreted form of eukaryotic of rLBP/p40 as assessed by the presence of V5 epitope on a Western blot. Equal amounts of total protein were loaded to lanes of SDS-page gels for each rLBP/p40 preparation.

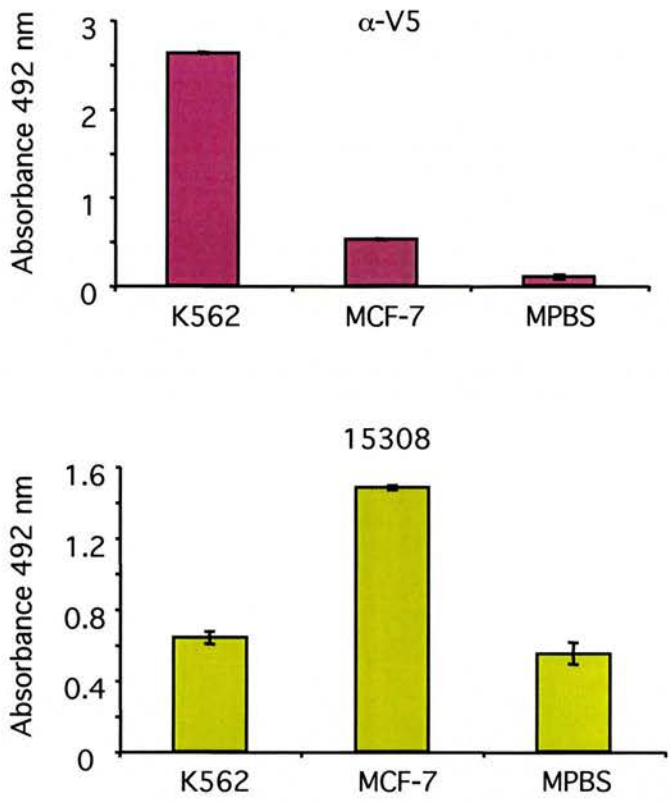


Figure 7-9. Comparison of Purified Preparations of Recombinant LBP/p40 (rLBP/p40) Expressed as Intracellular or Secreted Proteins by ELISA. rLBP/p40 was expressed as a polyhistidine and V5 tagged fusion protein either intracellularly in MCF-7 cells or in a secreted form in K562 cells and purified by nickel-metal affinity. Binding of either an anti-V5 antibody (top) or mAb 15308 (bottom) was tested on the purified preparations by ELISA. 2% milk powder in PBS (MPBS) was used as a control to assess the level of background binding for each antibody. Bound antibodies were detected via a goat anti-mouse peroxidase-conjugate as described in materials and methods. Data points are means +/- SD of duplicate wells in one ELISA.

Consideration of this along with examination of SDS–polyacrylamide gels stained for total protein (figure 7-7 panel C), showing strong bands at the corresponding molecular weights (~75 kDa and ~80 kDa) to the recombinant protein for K562 derived preparation and few contaminating proteins, it seems unlikely that the lack of reactivity by mAb15308 and mAb Lam-R was due to a lack of recombinant protein. Bearing in mind also, the observed increase in molecular weight for this preparation, one explanation is that additional modifications imparted by processing through the secretory pathway resulted in loss or masking of the epitope recognised by these antibodies.

7.2.4 Intracellular localisation of exogenous LBP/p40.

Two different constructs were used to analyse the localisation of exogenously expressed LBP/p40 within transfected mammalian cells; (1) LBP/p40 fused to a V5 epitope (see methods section 2.1.4) allowing detection with an anti-V5 antibody, and (2) LBP/p40 fused to green-fluorescent-protein (EGFP) (see methods section 2.1.6.1).

When detecting V5-tagged LBP/p40 using an antibody to the V5 epitope, diffuse staining was seen throughout the cytoplasm (figure 7-10 panel A-D). LBP/p40 expressed as an N-terminal EGFP fusion protein localised mainly to the perinuclear region, nucleus and, to a lesser extent in the cytoplasm (Figure 7-11).

For both the EGFP- and V5-tagged constructs, fluorescence-associated with the exogenously expressed LBP/p40 was not found to mirror endogenous 15308 labelling (i.e. filamentous, microtubule association), with colocalisation occasionally found restricted to the perinuclear region. Furthermore, transfection with exogenous LBP/p40 failed to generate a detectable increase in mAb15308 binding.

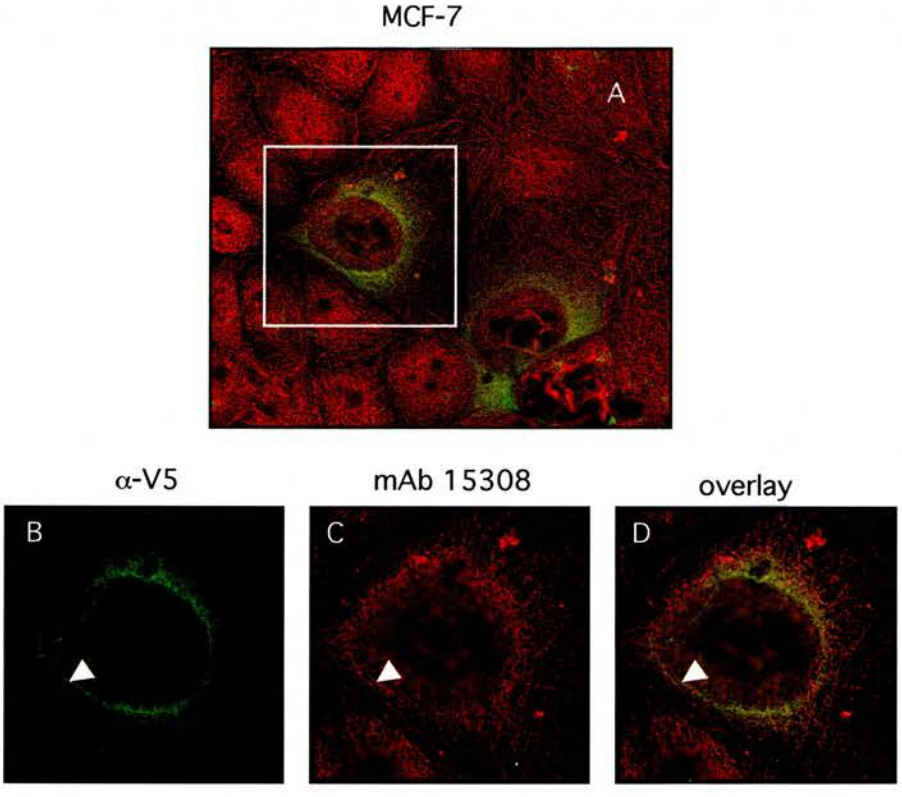


Figure 7-10. Intracellular localisation of recombinant LBP/p40 expressed as a C-terminal-V5-tagged fusion protein.
MCF-7 cells transiently transfected with pCDNA3.1D/LBP/p40-V5-His and double stained with mAb 15308 followed by AlexaFluor-568 labeled secondary antibody (red) and anti-V5 followed by AlexaFluor-488 labeled secondary antibody (green).
B-D Expanded view of image contained within the white box in A; arrows indicate an example of an area (albeit restricted) where double labelling was apparent.

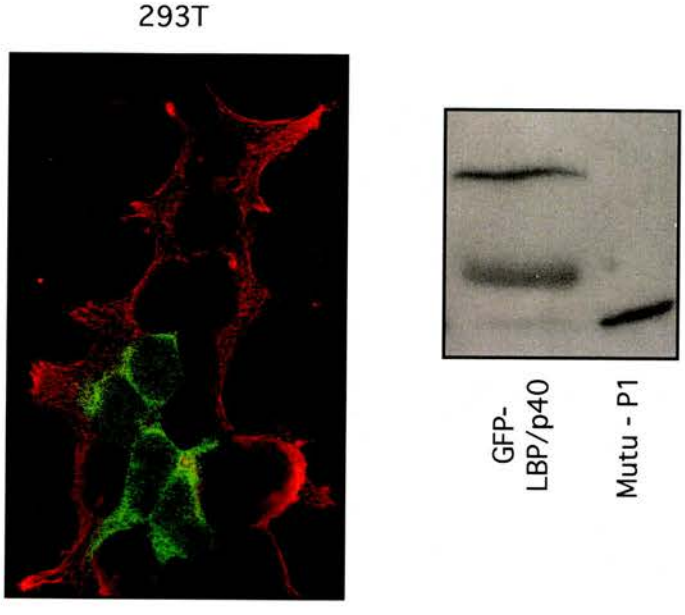


Figure 7-11. Intracellular localisation of recombinant LBP/p40 expressed as an N-terminal-EGFP fusion protein.
293T cells transiently transfected with pEGFP-C3-LBP/p40 (green) stained with mAb 15308 followed by AlexaFluor-568-labeled secondary antibody (red). Fluorescence was assessed by confocal microscopy. Preservation of the epitope recognised by mAb 15308 in this construct was confirmed by Western blot analysis. The increase in molecular weight of reactive bands in GFP-LBP/p40 compared to endogenous LBP/p40 (seen clearly in Mutu P1 fraction included as a positive control for mAb 15308 reactivity, c.f. section 7.2.1.1) being due to the EGFP tag.

There are a number of possible explanations for these observations. It could be that the epitope recognised by mAb 15308 in LBP/p40 on a denaturing gel by Western blot is hidden in LBP/p40 in its native, folded form within intact cells. This would require there to be a different epitope for recognition by the antibody as seen by microscopy. Alternatively, exogenously expressed LBP/p40 may not always be processed in the same way as the endogenously expressed protein resulting in the inability of mAb 15308 to bind the protein. In addition, there is the possibility that the tags provided by the expression vectors could interfere with sequence-specific localisation signals for LBP/p40. Evidence that mAb 15308 can indeed recognise LBP/p40 in its native form (as opposed to the denaturing conditions used for Western blot analysis) will be presented in section 7.2.7.1.2.

7.2.5 MAb 15308 does not bind to the 67kDa cell-surface expressed form of LBP/p40

A 67kDa form of LBP/p40 is expressed on the surface of a number of healthy cells and elevated levels on tumour cells (Menard, Tagliabue et al. 1998; Canfield and Khakoo 1999; Montuori, Selleri et al. 1999). MLuc5 is a monoclonal antibody produced by immunisation with a human small cell lung carcinoma cell line, originally characterised by its ability to immunoprecipitate a 67 kDa molecule from the membrane of the laminin-binding target cells (Martignone, Pellegrini et al. 1992). This antibody specifically recognises the 67 kDa, but not the 37 kDa form of LBP/p40. Surface staining of viable MCF-7 cells with MLuc5 confirmed expression of the 67kDa form in this cell line (figure 7-12).

However, no surface staining of viable MCF-7 cells was seen with mAb 15308 suggesting that the epitope recognised by this antibody on the 67 kDa surface form on viable cells is either on an intracellular portion of the molecule, hidden on an extracellular portion of the molecule due to the additional modifications required to process the 37 kDa form to the 67 kDa form, or totally absent from this isoform.

In addition, a commercially available antibody to LBP/p40 (Lam-R) that recognises the same proteins as 15308 by Western blot (figure 7-6) also failed to detect the epitope seen by Mluc5. Mutu I, BJAB, K562, 293T and A549 cells were all negative for the epitope on the 67kDa form of LBP/p40 recognised by MLuc5 (see appendix A7-1 for examples).

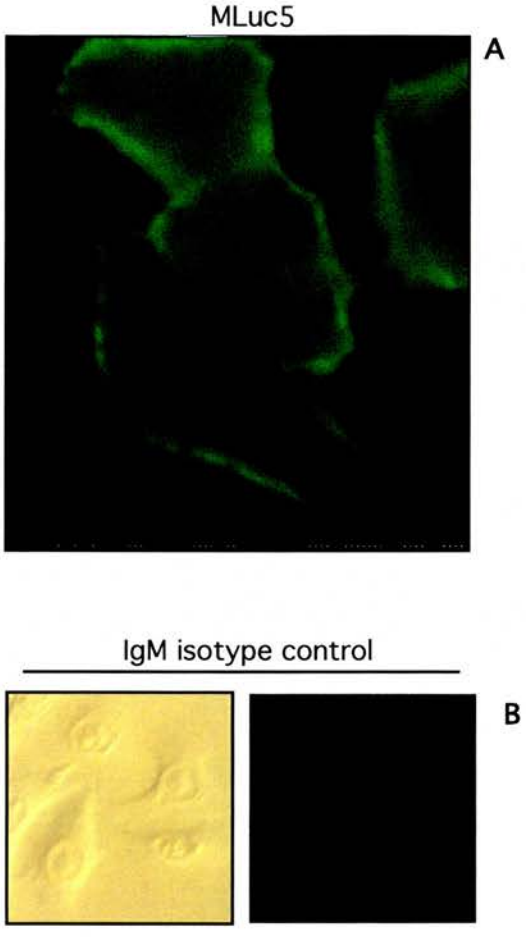


Figure 7-12. Surface expression of the 67kDa form of LBP/p40 on viable MCF-7 cells as assessed by standard fluorescence microscopy. **A;** Viable MCF-7 cells were probed with mAb MLuc5 (anti-67kDa LBP/p40) followed by FITC-labeled secondary antibody. **B;** No staining was observed with isotype matched antibody to MLuc5 (IgM)

7.2.6 Surface expression of the 15308 epitope during apoptosis.

7.2.6.1 Surface expression on MCF-7 and Mutu I cells

Results chapter 3 presented data showing that the epitope recognised by mAb 15308 can be found associated with cells undergoing apoptosis. During these initial studies with apoptotic Mutu I cells it became apparent that cells staining positive for mAb 15308 were at a relatively late phase of apoptosis. Thus, due to the preparation required for immunofluorescence analysis of such fragile suspension cultures, it was difficult to ascertain whether these cells had lost their membrane integrity. To study whether the epitope defined by mAb 15308 can be exposed on the surface of apoptotic cells prior to loss of membrane integrity, a system that imposes lower shear forces than those applied to flow cytometry preparations was required. To achieve this, apoptosis was induced in the adherent cell line, MCF-7, with etoposide for 48-72 hours and immunofluorescent staining by mAb 15308 analysed by confocal laser-scanning microscopy. As shown in figure 7-13 some cells could be identified unequivocally as positive for surface staining under these conditions.

Surface 15308 reactivity was mostly localised to areas corresponding to the blebs and apoptotic bodies. Lack of staining with PI demonstrated that the membrane covering the main body of these cells was intact (although not necessarily true for individual blebs), in contrast to PI-positive "leaky" cells (figure 7-13 panels C-E arrow). No surface staining was seen with the same population of apoptotic cells incubated with isotype control antibodies or with an antibody to β-actin (appendix A7-2).

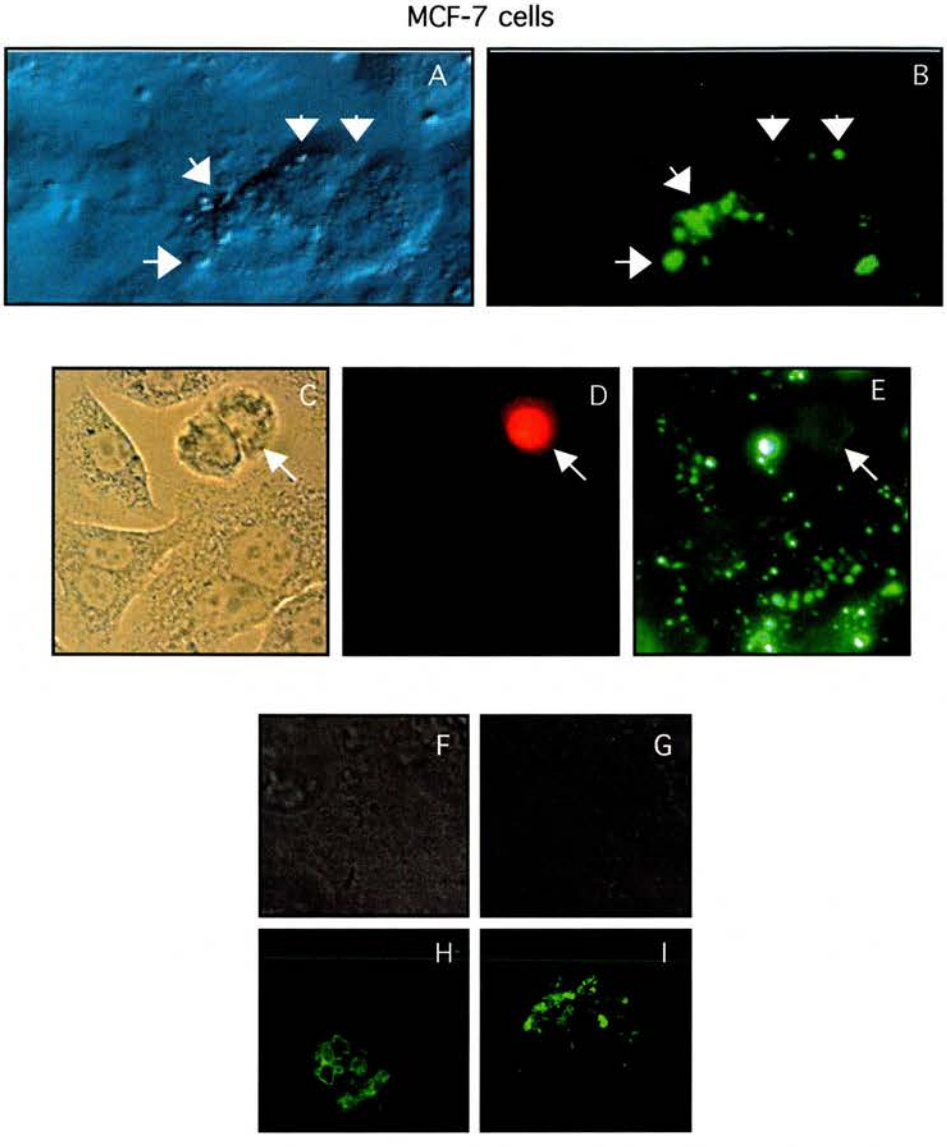


Figure 7-13. The 15308 epitope is exposed on the surface blebs of apoptotic MCF-7 cells, as determined by microscopy.
MCF-7 cells were treated with etoposide for 48-72 hours to undergo apoptosis. Binding of the anti-LPS antibody, 15308 was detected with goat anti-mouse secondary antibody labeled with AlexaFluor-488 (green). PI (red) was included in all samples as a measure of plasma membrane integrity throughout the procedure and to exclude the possibility that the 15308 antibody bound intracellular epitopes instead of cell surface-exposed molecules. A-E: assessment by standard microscopy (panels A-B and C-E are from two separate fields of view). The 15308 antibody stained bleb-like regions on the cell-surface of some of the apoptotic cells (examples indicated with arrow heads). F-I Closer inspection of surface blebs of two separate cells by confocal microscopy.

In order to test cell types and induction agents other than etoposide-treated MCF-7 cells for the capacity to expose this epitope at the cell surface, the decision was made to return to Mutu I cells induced into apoptosis with ionomycin using a modified staining protocol involving fewer, gentler wash steps and shorter antibody incubation times to reduce the overall stress on the fragile plasma membranes of the apoptotic cells. Flow cytometric analysis of cells stained in this way showed that a subpopulation of cells defined by light scattering properties (Dive, Gregory et al. 1992) to be at a late phase of apoptosis bound mAb 15308 whilst remaining negative for the uptake of PI (figure 7-14). As previously established, no staining was observed with either viable/early apoptotic cells or the population frequently observed using this modified protocol that appeared in-between the two regions (figure 7-14 panels labelled R1 and R2). The isotype-matched control antibody γ3 displayed no staining.

Confocal microscopic analysis revealed that, consistent with the observations on MCF-7 cells, staining appeared to be confined to the surface blebs of non-leaky cells or polarised to one region of the surface (figure 7-15). Staining of surface blebs was also observed for apoptotic BJAB cells (figure 7-16). The appearance of 15308 epitope was inhibited by caspase inhibitor Z-BOC-fmk (figure 7-17), confirming that the event was caspase-dependent and therefore likely to be associated with an apoptotic process and not simply a response to stress induced by the presence of ionomycin.

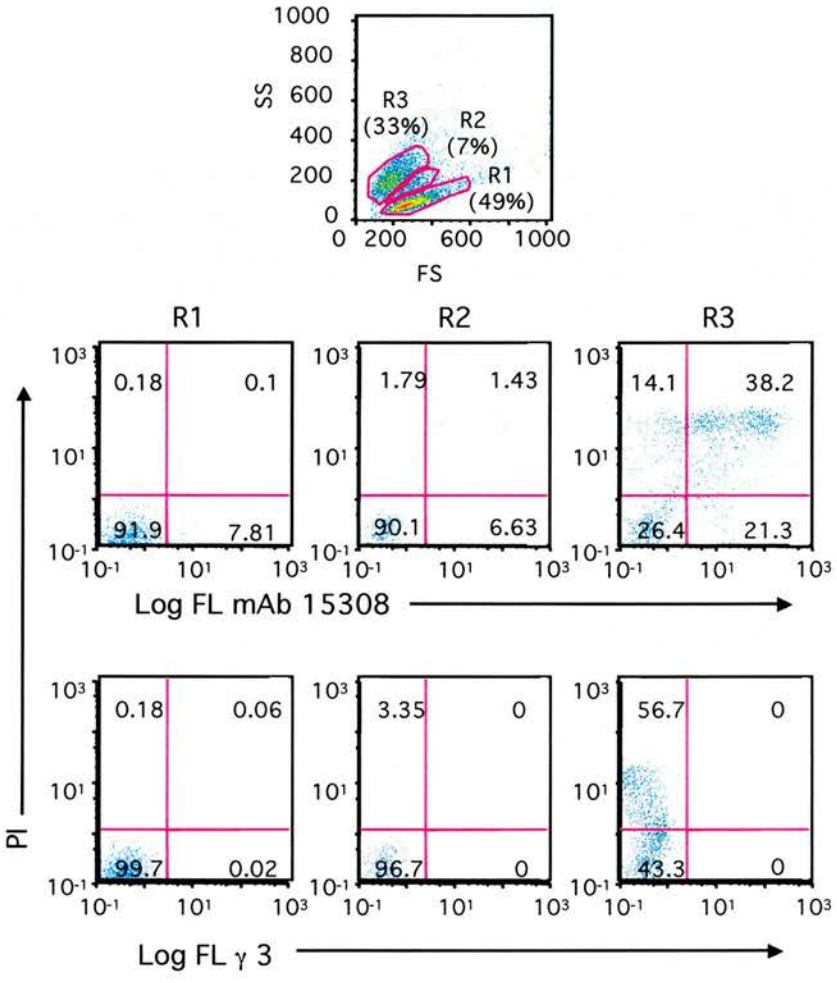


Figure 7-14. The 15308 epitope can be observed associated with apoptotic cells that are negative for the uptake of vital dyes as determined by flow cytometry.

Mutu I cells treated with ionomycin for 14 hours to undergo apoptosis were assessed for binding of the anti-LPS antibody, 15308 compared to isotype-matched control (γ3). Bound antibody was detected with goat anti-mouse secondary antibody labeled with FITC. PI was included as a measure the membrane integrity. Within the population, cells displayed light scattering properties corresponding to being either viable/early apoptotic (R1), late apoptotic (R3) or at an in-between phase (R2) (see methods section 2.3.3.3)

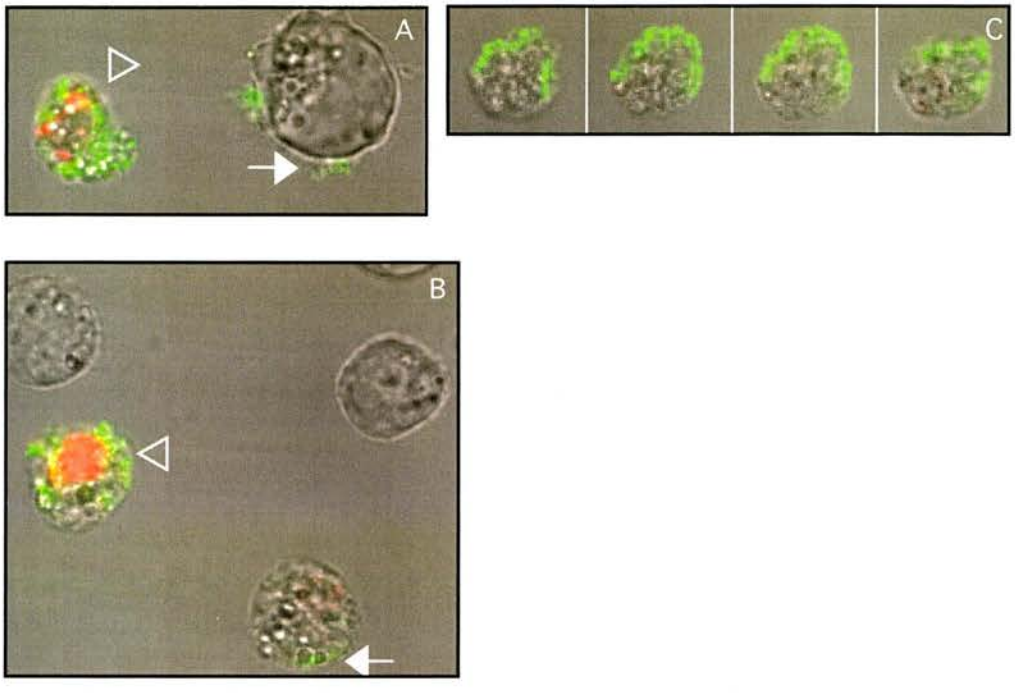


Figure 7-15. The 15308 epitope is exposed on the surface of apoptotic human lymphoma cells, as determined by confocal microscopy.

The human B-cell lymphoma cell line, Mutu I was treated with ionomycin for 16 hours to undergo apoptosis. Binding of the anti-LPS antibody, 15308 was detected with goat anti-mouse secondary antibody labeled with AlexaFluor-488 (green). PI (red) was included in all samples as a measure the integrity of the membrane throughout the procedure and to exclude the possibility that the 15308 antibody bound intracellular epitopes instead of cell surface-exposed molecules.

As seen in A and B, the 15308 antibody stained the cell surface of some of the apoptotic cells (closed arrow heads), whereas secondarily necrotic cells (open arrow heads) exhibited nuclear staining with PI and cytoplasmic staining.

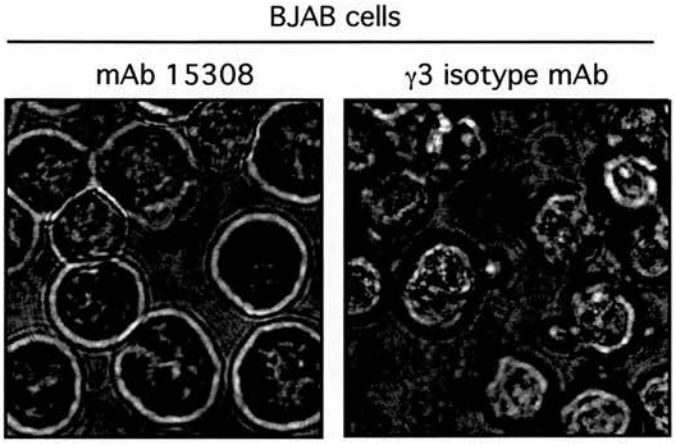


Figure 7-16. The 15308 epitope is also exposed on the surface of BJAB cells, as determined by confocal microscopy.
The human B-cell lymphoma cell line, BAJB was treated with ionomycin for 16 hours to undergo apoptosis. Binding of the anti-LPS antibody, 15308 was detected with goat anti-mouse secondary antibody labeled with AlexaFluor-568 (red). TO-PRO-3 dye (which would appear blue) was included in samples as a measure the integrity of the membrane throughout the procedure and to exclude the possibility that the 15308 antibody bound intracellular epitopes instead of cell surface-exposed molecules. No staining was observed with an isotype-matched antibody to mAb 15308 (γ3).

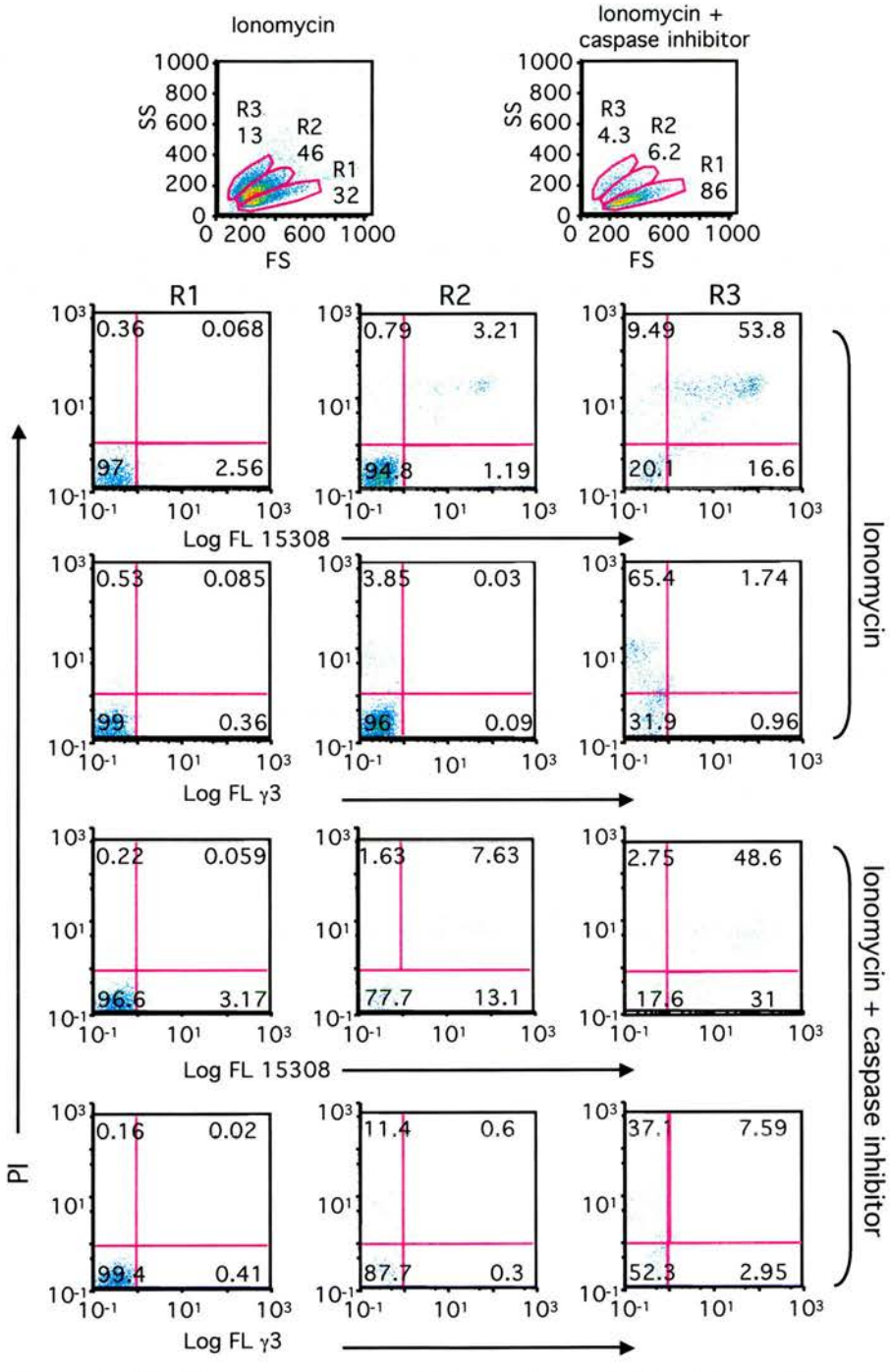


Figure 7-17. The appearance of the epitope defined by mAb 15308 on PI negative ionomycin treated cells is caspase dependent as determined by flow cytometry. Mutu I cells treated with ionomycin in the presence or absence of the caspase inhibitor Z-BOC-FMK (50μM) for 14 hours before being assessed for binding of the anti-LPS antibody, 15308. Bound antibody was detected with goat anti-mouse secondary antibody labeled with FITC. PI was included as a measure the membrane integrity. Within the population, cells displayed light scattering properties corresponding to being either viable/early apoptotic (R1), late apoptotic(R3) or at an in-between phase (R2) (see methods section 2.3.3.3).

7.2.6.2 Co-distribution of the epitope defined by mAb 15308 with other known apoptotic-cell markers

Numerous molecules have been shown localised to the bleb-like structures that arise at the cell-surface during apoptosis (Cocca, Cline et al. 2002) (Korb and Ahearn 1997). To further investigate whether the cell-surface appearance of the epitope recognised by mAb 15308 is in agreement with such markers, co-distribution was explored with two indicators of apoptotic-cell blebs, Annexin V and C1q.

Confocal analysis demonstrated co-distribution of mAb 15308 and Annexin V binding to isolated parts of cell surfaces and blebs of unpermeabilised, etoposide-treated MCF-7 cells (Figure 7-18). Although both markers seemed to be confined to the same regions of the cell surface, complete colocalisation (judged by the appearance of golden fluorescence) was only occasionally observed. Similarly, when cells were co-incubated with mAb 15308 and biotinylated, purified C1q, then stained with the relevant fluorochrome-conjugated reagents for detection, regions of cells that stained positive for mAb 15308 also stained positive for C1q (figure 7-19). As was noted from co-staining with Annexin V, colocalisation was occasionally observed around blebs and polar-regions on the cell surface. No staining was observed when biotinylated IgG1 was used instead of C1q as an irrelevant protein control or when cells were stained with an isotype-matched irrelevant antibody for mAb 15308 (appendix A7-3).

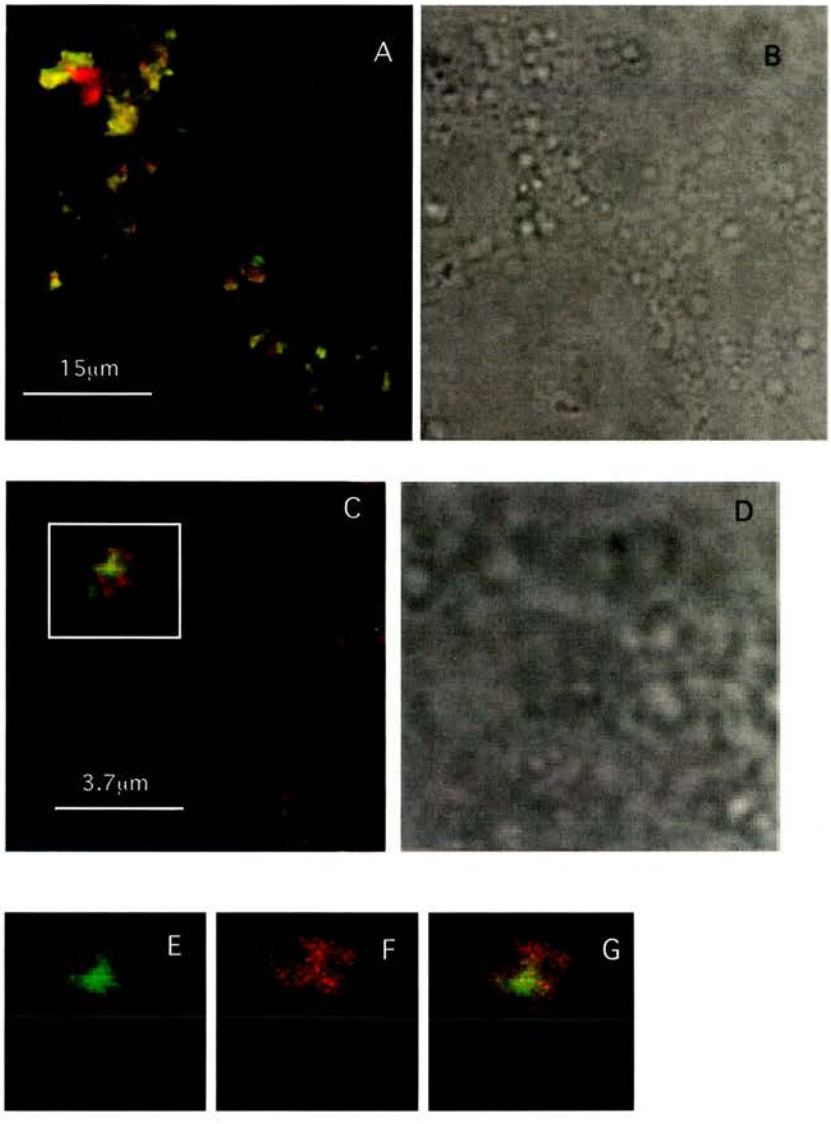


Figure 7-18. Colocalisation of 15308-reactivity and PS on the surface of apoptotic human breast carcinoma cells.
MCF-7 cells were treated with etoposide (100μM) for 48 hours, and the colocalisation of the 15308 epitope and PS examined. Shown are the confocal fluorescence images of 15308 epitope visualised by goat anti-mouse secondary antibody labeled with AlexaFluor-568 (red), biotinylated-annexin V binding to PS visualised by streptavidin labeled with AlexaFluor-488 (green). A and B: The epitopes recognised by mAb 15308 and Annexin V localise to regions containing bleb like structures. E,F and G: Close up view showing staining of bleb surface (white square C) with single Z-section images. B and D: Bright field. A,C and G: Dual excitation for colocalisation.

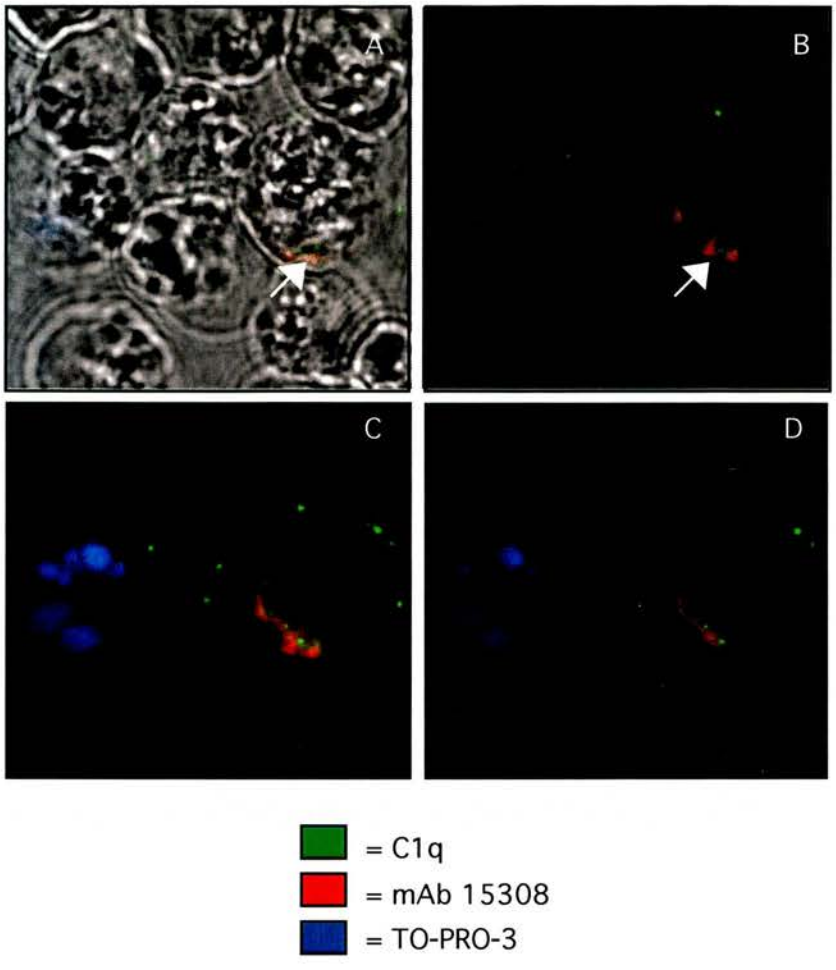


Figure 7-19. Colocalisation of 15308 and C1q reactivity on apoptotic Mutu I cells as assessed by confocal microscopy.
Mutu I cells were treated with ionomycin for 16 hours to undergo apoptosis, before examining the localisation of the epitopes recognised by mAb 15308 and biotinylated-C1q. Bound mAb 15308 was visualised using a goat anti-mouse secondary antibody labeled with AlexaFluor-568 (red), biotinylated C1q was visualised using streptavidin labeled with AlexaFluor-488 (green). TO-PRO-3 (blue) was included in all samples as a measure of membrane integrity. A; bright field image overlayed with the fluorescence associated with a mid-way Z-section (fluorescence alone shown in B) demonstrating co-distribution of mAb 15308 and C1q. C; total cell-associated fluorescence. D; fluorescence associated with a different z-section from that displayed in A and B. No staining was observed with isotype matched antibody to 15308 or an irrelevant biotinylated protein (appendix 7-3).

7.2.6.3 Surface expression of exogenously expressed LBP/p40 during apoptosis.

In an attempt to determine whether LBP/p40 is the (or one of the) molecule(s) bearing the epitope recognised by mAb 15308 displayed on the cell-surface during apoptosis, the presence of exogenously-expressed cell-surface LBP/p40 was examined following apoptosis induction. For these studies, two cell lines stably expressing the molecule were examined. Firstly, MCF-7 cells expressing V5-tagged LBP/p40 and secondly BJAB cells stably expressing EGFP-tagged LBP/p40. Figures 7-20 and 7-21 display example images of each analyses.

As noted for EGFP-tagged LBP/p40 293T transfectants in section 7.2.4, in contrast to MCF-7 transfectants which exhibited diffuse cytoplasmic staining with an antibody to V5-tagged LBP/p40, viable BJAB transfectants exhibited a significant level of nuclear-associated LBP/p40 (figure 7-21). No detectable increase in the level of surface staining with mAb 15308 was observed with EGFP-tagged LBP/p40-transfected cells compared to wild-types during apoptosis.

In the case of MCF-7 cells induced into apoptosis with etoposide it was possible to detect small amounts of the V5 epitope at the surface of non-membrane-compromised cells. However the appearance of this exogenously expressed LBP/p40, was a scarce event (figure 7-20, $< 2\%$ of total cells). One could interpret these results in a number of ways. Firstly, it could be that LBP/p40 is not the molecule recognised by mAb 15308 at the cell-surface during apoptosis. Secondly, it is possible that LBP/p40-transfectants are unable to translocate exogenous LBP/p40 to the cell-surface during apoptosis as efficiently as endogenous LBP/p40.

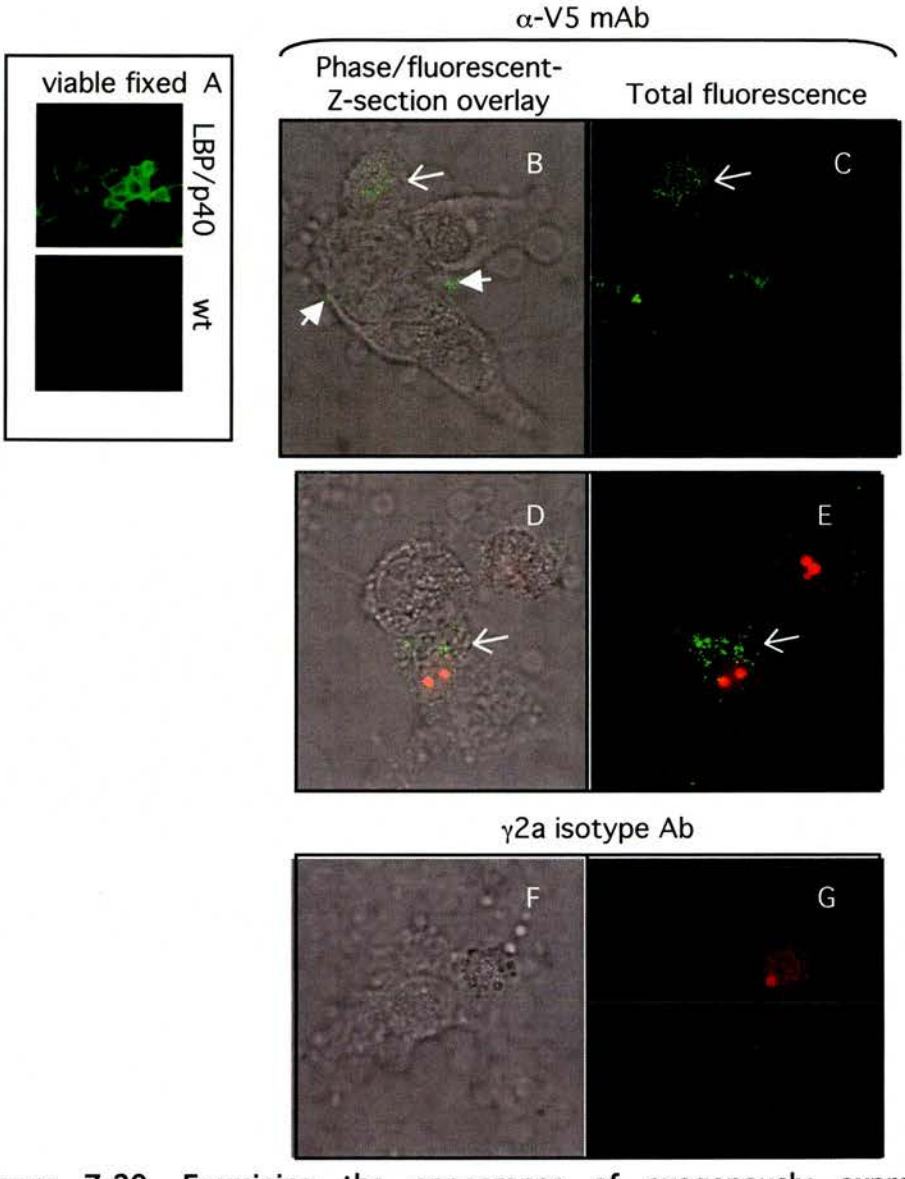


Figure 7-20. Examining the appearance of exogenously expressed LBP/p40 on the surface of apoptotic MCF-7 cells by confocal microscopy. **A;** The expression of V5-tagged LBP/p40 in transfected cells in comparison to wild-type non-transfected (wt) was assessed following permeabilisation of viable cell monolayers by staining with an antibody to the V5 epitope. Bound antibody was detected with goat anti-mouse secondary antibody labeled with alexaFluor-488 (green). **B-G;** MCF-7 cells stably expressing V5-tagged LBP/p40 were treated with etoposide (100μM) for 48 hours prior to staining. PI (red) was included in all samples as a measure the integrity of the membrane throughout the procedure. On rare occasions (<2% total cells) examination of individual Z-sections revealed the presence of the V5 epitope on surface blebs of membrane intact cells (closed arrows in B) as opposed to membrane compromised cells (open arrow B, D) which stained throughout the cell and were positive for PI (seen more clearly through examination of total cell fluorescence, open arrows C, E).

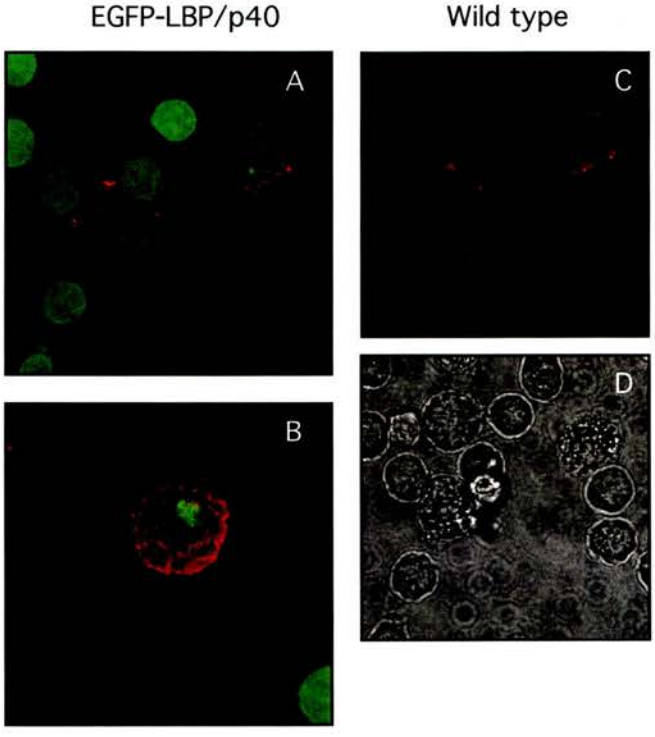


Figure 7-21. Examining the appearance of mAb 15308-reactivity on the surface of EGFP-tagged BJAB transfectants.
BJAB cells were treated with ionomycin for 16 hours to undergo apoptosis before staining with mAb 15308. Bound antibody was visualised using a goat anti-mouse secondary antibody labeled with AlexaFluor-568 (red). TO-PRO-3 was included in all samples (although not shown in these images) to allow discrimination between membrane intact (panel A) and membrane compromised mAb 15308-positive cells. No detectable increase in surface staining with mAb 15308 was observed in EGFP-tagged LBP/p40 transfected cells (green A and B) as compared to wild type cells (C and D).

Finally, it could be that LBP/p40 is processed in the same way as the molecule(s) bearing the endogenously expressed epitope(s) recognised by mAb 15308, but at levels at the lower end of detection limits.

7.2.7 Functional studies involving LBP/p40

7.2.7.1 Binding of recombinant LBP/p40 to CD14

7.2.7.1.1 Initial studies utilising mammalian and bacterial cell-derived recombinant LBP/p40

Initially, purified recombinant LBP/p40 (rLBP/p40) derived from the two previously described mammalian expression systems (sections 7.2.3.3 and 7.2.3.4) and the bacterial system (section 7.2.3.2) were assessed for preferential binding to CD14-transfected K562 cells compared to CD14 negative cells. The nickel metal-affinity purified preparations of rLBP/p40 were allowed to incubate with cells and bound protein subsequently detected with α-V5 antibody by flow cytometry. Whereas binding of LBP/p40 preparations derived from the culture supernatants of transfected K562 cells or lysates of transfected MCF-7 cells did not display binding, preparations from the bacterial expression system bound preferentially to CD14-expressing cells compared to CD14-negative cells (figure 7-22). Interpretation of these findings must be made with caution as subsequent analysis showed LPS to be present in high amounts in the bacterially derived preparation (>250 endotoxin units/ml). However, when binding experiments were carried using the preparations derived from mammalian expression systems in the presence of comparable levels of purified LPS from *Pseudomonas aeruginosa* (based on the assumption that 10

endotoxin units of in a chromogenic Limulus amoebocyte lysate (LAL) assay is equivalent to 1ng LPS), binding of these preparations to CD14-expressing K562 cells was not enhanced (figure 7-23).

Attempts to remove LPS (achieving <0.5 endotoxin units/ml) from the bacterial-derived preparation also led to a significant loss of protein (10 fold reduction) and consequently a reduction in binding to CD14 expressing K562 cells (figure 7-23, bottom panels). Given the hypothesis that LBP/p40 bears an "LPS-like" epitope, any approach to remove LPS from LBP/p40 using affinity-based endotoxin-specific decontamination systems might be flawed from the outset.

Given these inconclusive observations, attempts were made to obtain a sufficient quantity of purified "LPS-free" LBP/P40 in order to confirm or refute the capacity of the molecule to interact with CD14. This was achieved by infection of sf9 insect cells with a LBP/p40-recombinant Baculovirus vector, the results of which are detailed in the following section.

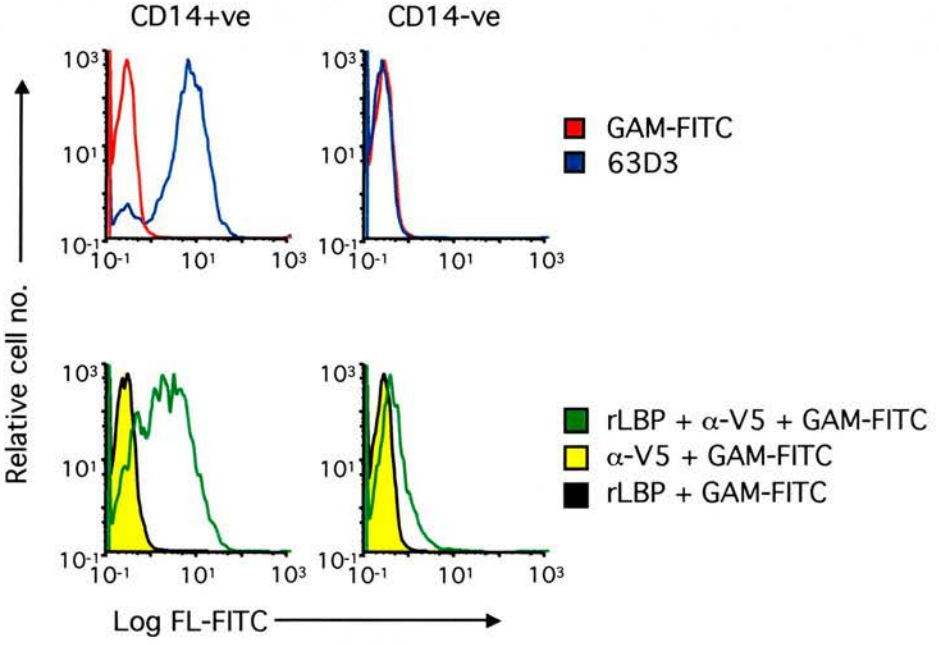


Figure 7-22. Purified recombinant LBP/p40 (rLBP/p40) derived from a bacterial expression system binds preferentially to CD14 expressing cells.

Purified preparations of rLBP/p40 were incubated with either wild type K562 cells (CD14 -ve) or K562 cells overexpressing CD14 (CD14 +ve). Bound recombinant protein was was detected with an antibody to the V5 tag of rLBP/p40 (α-V5) followed by an anti-mouse secondary antibody labeled with FITC. The expression of CD14 on cells was confirmed by staining with mAb 63D3 (anti-CD14).

Representative of 4 staining experiments using preparations of rLBP/p40 produced on two independent occasions.

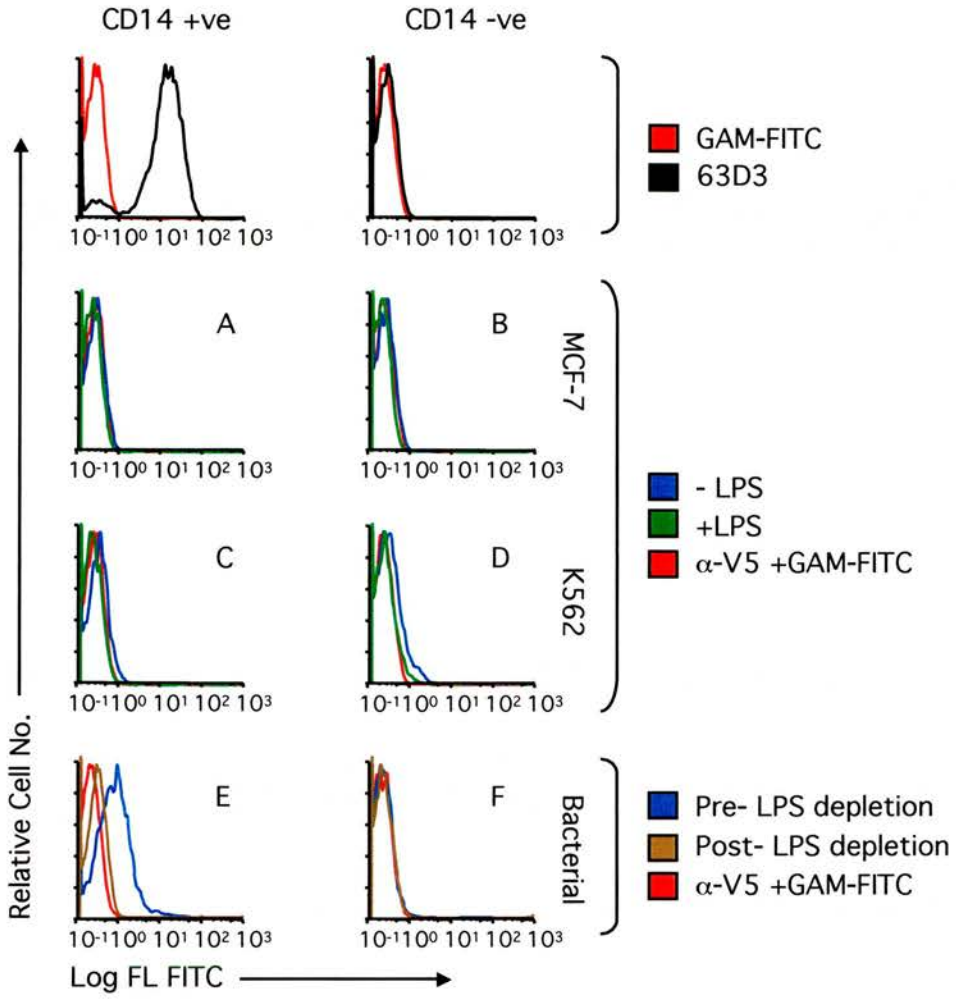


Figure 7-23. Effect of LPS contamination on the ability of recombinant LBP/p40 (rLBP/p40) to interact preferentially with K562 cells expressing CD14.

Purified preparations of rLBP/p40 were incubated with either wild type K562 cells (CD14 -ve) or K562 cells overexpressing CD14 (CD14 +ve). **A-D**; The addition of comparable amounts of LPS found to that found in the bacterial derived preparation of LBP/p40 (green histograms) to preparations of LBP/p40 derived from endotoxin-free mammalian cell expression systems (MCF-7 or K562) did not enhance binding to CD14 (blue histograms). **E,F**; Conversely, depletion of LPS (brown histograms) from bacterial-cell derived LBP/p40 preparations resulted in reduced interaction with CD14. However the process of LPS depletion also led to a reduction in protein concentration within the preparation, and thus a possible cause for the reduced signal.

Bound recombinant protein was was detected with an antibody to the V5 tag of rLBP/p40 (α-V5) followed by an anti-mouse secondary antibody labeled with FITC. The expression of CD14 on cells was confirmed by staining with mAb 63D3 (anti-CD14).

7.2.7.1.2 Production and Purification of LBP/p40 in insect cells using a Baculovirus expression system.

Western blot analysis of Sf9 cells infected with recombinant LBP/p40 baculovirus revealed the presence of relatively large amounts of mAb 15308-reactive protein (figure 7-24, panel A). In contrast to the crude cell-lysates obtained from mammalian and bacterial cells exogenously expressing LBP/p40, a clear band was visible on a total protein-stained SDS-polyacrylamide gel compared to non-transfected/transformed cells (figure 7-24 panel B), corroborative of high-level expression.

The high level of expression of LBP/p40 in sf9 cells was used to confirm that this protein in its native form can be recognised by mAb 15308 by ELISA (a concept that was questioned following investigation of the intracellular localisation of exogenously expressed LBP/p40 in section 7.2.4). Thus, a signal was seen with mAb 15308 when lysates from infected cells were used to coat ELISA plated compared with lysates from non-infected cells coated with matching concentrations of total protein (figure 7-25).

A strong signal was also seen by Western blot analysis for nickel-metal purified LBP/p40 from baculorvirus-infected cells with major and minor bands migrating at the expected molecular mass (strong band 50-70 kDa and a weaker band ~40 kDa figure 7-24, lane labelled p40/F). This construct was used in its partially purified form for binding assays, to CD14-expressing cells K562. As was the case for LBP/p40 expressed in bacterial and mammalian expression systems, Baculoviral-expressed LBP/p40 was tagged at the C-terminus with a V5 epitope. Due to

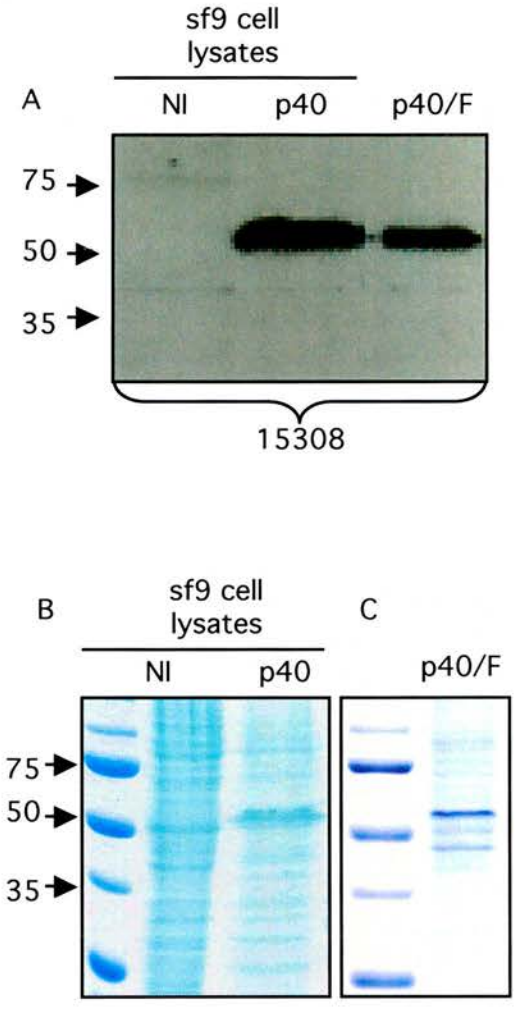


Figure 7-24. Expression of the recombinant LBP/p40 (rLBP/p40) in baculovirus-infected sf9 insect cells.

A; The presence of rLBP/p40 in sf9 insect cells detected by probing Western blots of total protein from non-infected (NI) or recombinant-LBP/p40 (p40) baculovirus-infected cells with mAb 15308.

The level of expression in crude cell-lysates (B) and degree of purity in nickel metal-affinity purified LBP/p40 (p40/F) (C) was assessed by examination of 10% SDS-polyacrylamide gels stained for total protein with GelCode-blue™ (B) or Coomassie blue (C).

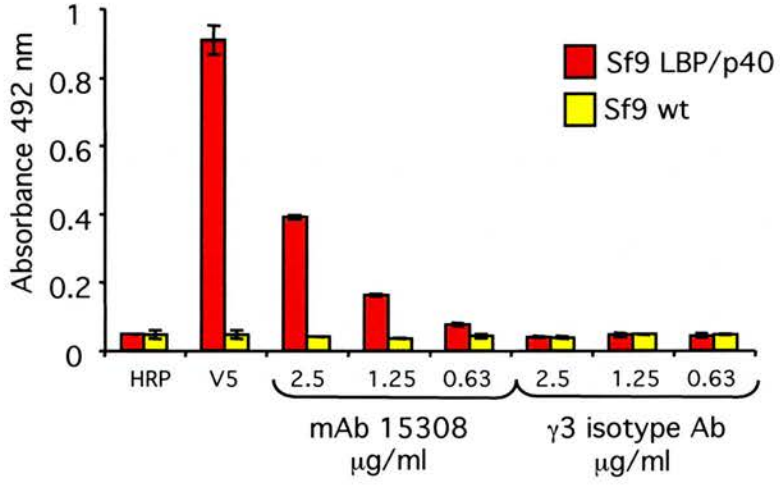


Figure 7-25. Binding of mAb 15308 to LBP/p40 derived from insect cells.

An ELISA against lysates prepared from sf9 insect cells infected with LBP/p40-recombinant Baculovirus (red bars) or left uninfected (yellow bars) was used to confirm the reactivity of mAb 15308 to LBP/p40 under non-denaturing conditions. γ3 isotype-matched antibody did not react with Baculovirus-infected cells. ELISA plates were coated with equal amounts of total protein from cell lysates.
Results shown are the mean +/- S.D. of duplicate wells.

technical difficulties and time limitations it was not possible to express and purify a control construct (i.e. NEDD5) in Sf9 cells. As a substitute negative control, nickel-metal purified soluble-LBP/p40 from K562 cells (which was shown not to contain the epitope recognised by mAb 15308, c.f. figure 7-6) was used for these studies at an equal concentration of α-V5 reactivity, as detected in a direct ELISA (Although it is appreciated that this is not an ideal control as it was expressed in a different system). The insect-cell-purified LBP/p40 construct clearly bound equally well to both CD14 positive and negative K562 cells (figure 7-26). This observation raises two issues; (1) the means by which LBP/p40 binds to K562 CD14 negative cells, and (2) the absence of preferential binding to CD14 positive cells.

Addressing the former question first; as mentioned in section 7.2.1.3 LBP/p40 is known to associate with α6 integrins (Ardini, Tagliabue et al. 1997). This could explain binding to CD14 negative K562 cells as the expression of some integrin chains by these cells has been documented (Denda, Reichardt et al. 1998). An alternative explanation is that K562 cells, being of myeloid origin are positive for a PRR other than CD14 that is capable of interacting with LBP/p40. However, this would not explain the ability of bacterial-cell derived LBP/p40 to bind preferentially to CD14 positive K562 cells.

In addressing the second issue, several explanations are possible for the absence of preferential binding by the insect-cell derived protein. One possibility is that preferential binding of the bacterial-derived protein was solely due to LPS contamination. This would require LBP/p40 to bind, either directly or indirectly to LPS, and for this complex to bind either directly or indirectly to CD14.

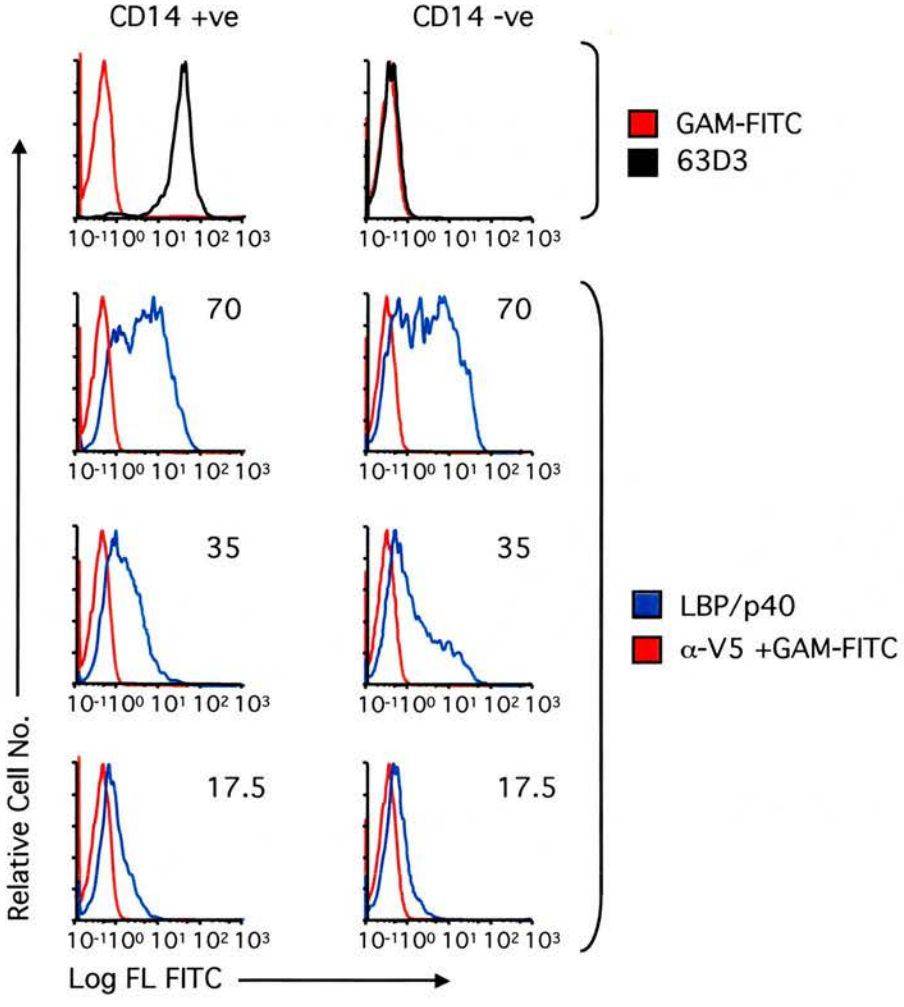


Figure 7-26. Purified recombinant LBP/p40 (rLBP/p40) derived from a recombinant-Baculovirus insect expression system does not bind preferentially to CD14-expressing cells.
Purified preparations of rLBP/p40 were incubated with either wild type K562 cells (CD14 -ve) or K562 cells overexpressing CD14 (CD14 +ve). Numbers at the top right hand corner of each histogram represent the total protein concentration (i.e. including contaminants) of rLBP/p40 incubated with cells (μg/ml).
Bound recombinant protein was detected with an antibody to the V5 tag of rLBP/p40 (α-V5) followed by an anti-mouse secondary antibody labeled with FITC. The expression of CD14 on cells was confirmed by staining with mAb 63D3 (anti-CD14).

An alternative explanation (which assumes that LBP/p40 contains a functional "LPS-like" pattern), is that binding of bacterial-cell derived LBP/p40 was facilitated by an LPS-binding protein present in the bacterial preparation not found in the sf9 cell-derived preparation. This proposition is based on the knowledge that LPS itself is rarely ever found in isolation, but is usually associated with a binding partner (Chaby 2004).

Studies intended to explore these issues included: -

- The addition of LPS to insect-cell derived LBP/p40 to test for enhancement of its binding to CD14-expressing K562 cells.
- Testing the ability of insect-cell derived LBP/p40 to interact preferentially with CD14 expressing cells of non-myeloid origin.
- Measuring the potential for preferential interaction between LBP/p40 to CD14 or other LPS-binding proteins by ELISA.

However, due to the instability of purified LBP/p40 preparations following freeze-thawing or prolonged storage at 4°C, and a tendency to precipitate from solution these tests were not completed at the time of documenting this thesis.

7.2.7.1.3 Point of contact between surface 15308 epitope and phagocyte membrane

As a starting point to investigate the role of LBP/p40 as a possible apoptotic-cell-associated ligand for macrophage recognition the relative coincidence of 15308-reactivity at the point of phagocyte interaction was examined. Confocal analysis of macrophage-apoptotic cell interaction with respect to mAb 15308 staining following a binding assay carried out at room temperature showed that the epitope recognised on the Mutu I cell-surface was occasionally localised at the sites where the cells attached to the mouse peritoneal-derived macrophages (summarised in figure 7-27). Interaction with membrane intact feed cells was only observed following very gentle washing, in contrast to thorough washing commonly used following interaction assays (c.f. section 6.2.2). Furthermore, even with such mild washing these were rare events (<5% of total mAb 15308-positive cells were non-leaky), and mAb 15308 un-reactive feed cells were also observed (figure 7-27, panel B, open arrows).

Preliminary attempts to recreate these findings with HMDM looking for point of contact between CD14 indicated a requirement for further optimisation as only leaky cells were observed bound to the phagocytes. However, these observations indicate the possibility that apoptotic cells can be recognised by macrophages through regions positive for "LPS-like" epitopes.

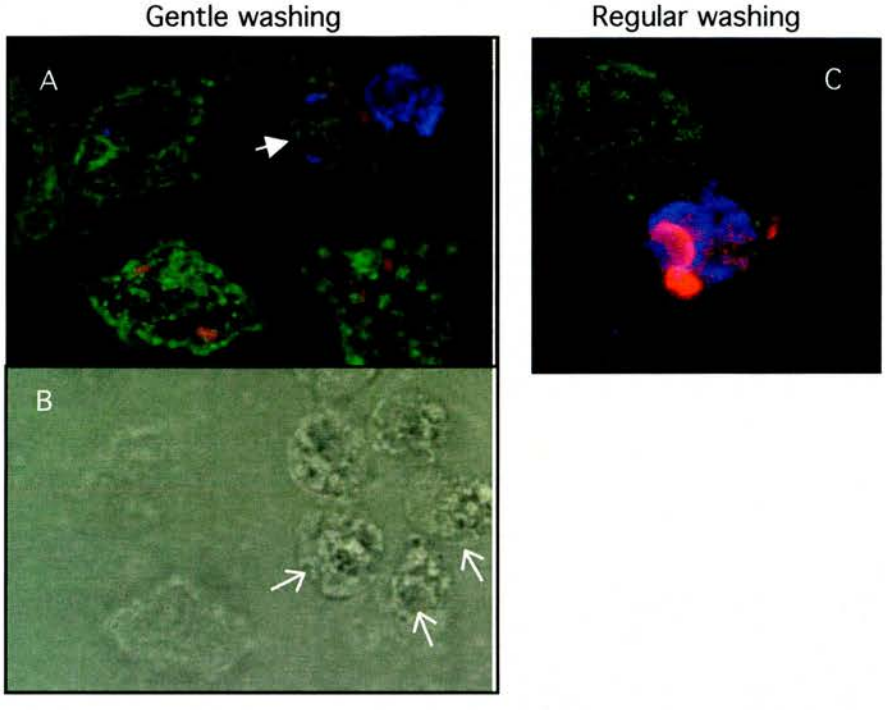


Figure 7-27. Localisation of mAb 15308 positive regions on apoptotic Mutu I cells at the point of contact with macrophages. After coincubation of apoptotic Mutu I cells with mouse peritoneal-derived macrophages at room temperature (as described in methods section 2.10) and following removal of unbound cells by either gentle or regular washing the cells were stained with mAb 15308 followed by secondary antibody labeled with alexaFluor-350 (blue), and AlexaFluor-488-conjugated mouse-F4/80 antibody. PI (red) was included in all samples as a measure membrane integrity.

Whilst A and C display total cell-associated fluorescence, scanning through sequential Z-sections revealed the appearance of the mAb 15308 positive regions (arrow in A) at the point of contact with the mouse macrophage membrane. Open arrows in B indicate mAb 15308-non-reactive feed cells. When regular, thorough wash steps were employed prior to staining, only membrane-compromised apoptotic feed cells were observed bound to macrophages (C).

The results described in this chapter can be summarised as follow: -

- MALDI/TOF spectrometrical analysis allowed identification of three candidate proteins with similar mobility to the mAb 15308-reactive band present in the electrophoretically-separated protein mixtures of Mutu I cells as:- (1) β-Actin, (2) Laminin binding protein (LBP/p40) and (3) NEDD5
- The ability of mAb 15308 to bind LBP/p40 exogenously expressed in HEK293T cells confirmed the observed cross-reactivity of this anti-LPS antibody.
- LBP/p40 was seen to exist in two main forms with molecular sizes of ~40 kDa and ~50-70 kDa when overexpressed as an intracellular protein in mammalian, bacterial and insect expression systems. In contrast, when expressed via the secretory pathway as a secreted protein in mammalian cells, LBP/p40 was found to be expressed as three main species of molecular weight ~50 kDa, ~75 kDa and ~80kDa.
- Whilst both mAb 15308 and Lam-R antibody were able to detect recombinant LBP/p40 expressed as an intracellular protein they did not recognise the secreted form.
- It was not possible to detect the exact co-distribution of mAb15308-reactivity with the localisation of exogenously expressed LBP/p40 within transfected mammalian cells by microscopy.
- As a result of apoptosis the epitope recognised by mAb 15308 can be detected at the cell-surface prior to loss of membrane integrity. Cell-surface

mAb 15308-reactivity was detected in the same localised regions bound by Annexin V and C1q. However, relatively lower amounts of exogenously expressed LBP/p40 were detected under the same conditions.

- Whilst partially-purified recombinant LBP/p40 derived from bacterial cells was observed to bind preferentially to CD14-expressing K562 cells compared to CD14 negative K562 cells, LBP/p40 derived from insect cells bound equally well to both cell types.

8 General Discussion

8.1 A Means to Define ACAMPs

The ability of anti-LPS antibodies to recognise structures associated with dying-self as demonstrated in this thesis increases the number of known classes of markers for apoptotic cells. Although it had been postulated previously that akin to the PAMPs of micro-organisms recognised by PRRs, apoptotic cells expose ACAMPs, and that the functioning of such phagocytic receptors in microbial recognition might have evolved secondarily to their function in the recognition of dying self (Franc, White et al. 1999) the criteria for defining ACAMPs have not been set. Thus far, all molecules on the surface of dying cells implicated in their clearance have been referred to as ACAMPs, including for example, markers such as CD31, which bear no obvious relationship to PAMPs.

While examples of individual innate PRRs interacting with both pathogens and apoptotic-cell-associated ligands including CD14, C1q and Pentraxin-3 (Devitt, Moffatt et al. 1998; Ogden, deCathelineau et al. 2001; Garlanda, Hirsch et al. 2002), have been described, the ACAMPs with which they interact have not. The results of the work described herein allow a clearer classification of ACAMPs in terms of molecular motifs shared between microbes and apoptotic cells (albeit in the absence of a functional definition at the time of writing).

8.2 New findings of "LPS-like" structures within and on apoptotic cells

The data presented in results chapter 3 established that viable eukaryotic cells hold epitopes that can be recognised by some antibodies previously characterised as having specificity towards lipopolysaccharide. In addition to this, the data in section 7.2.6 of results chapter 4 showed that these epitopes can be found on the surface of apoptotic cells prior to the loss of membrane integrity in the same localised regions to which Annexin V and C1q bind. Consistent with the majority of apoptotic-cell-surface markers defined previously, the "LPS-like" epitopes observed in these studies do not appear to have been generated as a result of the apoptosis process, as they were detected intracellularly in permeabilised viable cells of numerous types (section 6.2). Characterisation of previously defined apoptotic-cell-surface markers has revealed them to appear as a result of either changes to cell-surface resident molecules such as CD43 (Eda, Yamanaka et al. 2004), or from a redistribution of intracellular molecules to the cell surface such as Annexin 1 (Arur, Uche et al. 2003). The distribution of staining seen with mAb 15308 falls into the latter of these classes. The possible implications of these findings are discussed in section 8.4.

8.2.1 Identification of mAb 15308-reactive species present in Mutu I cells

MALDI/TOF mass spectrometry analysis of three candidate proteins for the species recognised by mAb 15308 within lysates of Mutu I cells revealed their identity as: - (1) β-Actin, (2) Laminin binding protein (LBP/p40), and (3) a Septin named NEDD5 (neuronally expressed, developmentaly downregulated-5). In section 7.2.2

the presence of cDNA encoding LBP/p40 and NEDD5 was detected from Mutu I mRNA by reverse transcriptase PCR. Following transfection of cDNA for LBP/p40 and NEDD5 into 293T cells, anti-LPS mAb 15308 was seen to bind exogenously expressed LBP/p40 but not NEDD5 suggesting that LBP/p40 bears an "LPS-like" epitope. Whilst investigating a potential for LBP/p40 to function in apoptotic-cell clearance, a number features characteristic to this molecule were noticed. The discussion will focus on these issues before considering possible future studies leading on from these findings.

8.2.1.1 Exogenously expressed, cloned LBP/p40 can be seen to exist as two major species on a denaturing gel

With the exception of expression as a secreted protein in K562 cells, exogenous expression of LBP/p40 resulted in the presence of two species of approximate molecular weight 40 kDa and 67 kDa (section 7.2.3). Studies aimed at elucidating the means by which the 67 kDa form of LBP/p40 is produced from its 37 kDa precursor suggest that it consists of homodimers of the lower molecular weight polypeptide (Landowski, Dratz et al. 1995). The 37 kDa polypeptide has been studied as a molecule restricted to the cytoplasm whereas the 67 kDa cell-surface expressed molecule, found on a limited number of healthy cells, is overexpressed on a number of malignancies (Yow, Wong et al. 1988; Rescan, Clement et al. 1991; al-Saleh, Delvenne et al. 1997; Menard, Tagliabue et al. 1998). The 37 kDa LBP/p40 is encoded by a single active gene (Jackers, Clausse et al. 1996; Jackers, Minoletti et al. 1996), the cDNA of which encodes a polypeptide precursor of ~300 amino acids,

which is post-translationally modified to the 67 kDa form (Castronovo, Claysmith et al. 1991). The complete process of this modification is not entirely clear but evidence suggests it requires cytoplasmic factors (Landowski, Dratz et al. 1995) (Buto, Tagliabue et al. 1998) (Castronovo, Claysmith et al. 1991). The ability of the dimer to withstand denaturing conditions such as those used for analysis in an SDS-polyacrylamide gel may be explained by the presence of a conserved lock-and-key structure/function domain at the carboxyterminal end of the protein (Clausse, Jackers et al. 1996). The fact that both forms of this protein were also observed when expressed in a bacterial system (section 7.2.3) provides information that eukaryote-specific processing is not required for this process.

Despite detecting the presence of a ~67 kDa protein by Western blot analysis of mammalian cell-lysates following transfection with LBP/p40 (section 7.2.3), surface expression with either Mluc5, α-LBP/p40, α-V5 or mAb 15308 was not detected on viable cells. Several studies investigating the requirements for surface expression have been published. To be expressed on the outer membrane, the 67 kDa form of the protein seems to require interaction with the α_6 integrin chain (Ardini, Tagliabue et al. 1997). This is supported by the observed coexpression and coimmunoprecipitation of the 67kDa LBP/p40 and α6 in tumour cells (Romanov, Sobel et al. 1994; Ardini, Tagliabue et al. 1997). In another study, transfection of 37 kDa LBP/p40 cDNA conferred 67 kDa LBP/p40 surface expression in a Jurkat clone that is usually negative for the 67 kDa protein. The authors suggested that this may be due to the fact that all these clones express some surface α_6 (Canfield and Khakoo 1999). However, in CHO cells, attempts to confer p67 kDa LBP/p40 surface expression by transfection of the 37 kDa LBP/p40 cDNA resulted in the

accumulation of cytoplasmic polypeptide (Castronovo, Claysmith et al. 1991). During the studies carried out for this thesis, transfection of the 37 kDa LBP/p40 cDNA into a cell line (MCF-7) capable of expressing the 67 kDa form endogenously, failed to detect an increase specifically in surface expression (section 7.2). If an accessory molecule (such as α6 integrin) is required to achieve this, one could argue that failure in increased surface expression may be due to a limitation in amounts of endogenous accessory molecule available.

Thus far, two forms of naturally occurring LBP/p40 have been mentioned, that is a 37 kDa intracellular form and a 67 kDa cell-surface form. However, the existence of an additional differentially processed form of this protein has been suggested by studies investigating a 37 kDa T-and B-lymphocyte-stimulating, tumor-specific rejection antigen, expressed on "the surface" of carcinomas, sarcomas and lymphomas/leukemias of rodents and humans which converged with studies of LBP/p40 (Coggin, Barsoum et al. 1999). The authors of this work reinforce that the molecule they refer to is not the 67 kDa form of LBP/p40 (Coggin, Rohrer et al. 2004) and have shown with both *in vitro* and *in vivo* studies that a monoclonal antibody specific for this 37 kDa "Oncofetal antigen (OFA)" detects the protein on various malignancies and in embryos/early fetuses but not in term fetus, neonate, or adult differentiated tissues (Coggin, Barsoum et al. 1998). OFA protein and 37kDa are > 99% identical proteins based on amino acid and cDNA sequencing (Coggin, Barsoum et al. 1999). OFA expression in the early foetus is in keeping with studies investigating a role for LBP/p40 in the developing limb bud (Hara, Satoh et al. 1997; Ruyani, Sudarwati et al. 2003). So it appears that the cell-surface expression of the 67 kDa form of LBP/p40 enables malignant tumour cells to penetrate laminin tissue

and vessel barriers, whereas the 37 kDa form of OFA-LBP/p40 activates anti-OFA-LBP/p40 specific cytotoxic T cells which can kill tumour targets. The relevance of differentially processed forms of LBP/p40 to the studies described in this thesis will be considered in the following section.

8.2.2 Intracellular localisation of exogenously-expressed LBP/p40.

Whilst demonstrating unambiguously the ability of mAb 15308 to bind LBP/p40 by Western blot analysis (section 7.2.3) and by ELISA (section 7.2.7), fluorescence microscopical comparison of exogenously expressed LBP/p40 and mAb 15308 reactivity detected differing distributions within cells (section 7.2.4). For both the EGFP- and V5- tagged LBP/p40 constructs, fluorescence associated with the exogenously expressed LBP/p40 was not found to mirror endogenous labelling with mAb 15308. Furthermore, transfection with exogenous LBP/p40 failed to cause a significant detectable increase in 15308 binding.

These discrepancies could be due to differential processing of the protein akin to those mentioned in the previous section. As mentioned in section 7.2.4, there are several other possible explanations for these observations. One explanation is that differing sensitivity of mAb15308 and anti-V5 (for exogenous LBP/p40) make comparison of the different forms difficult. Thus, if the antibody to the V5 tag produces a much higher signal (as demonstrated by Western blot analysis) then perhaps an increase in signal would not be seen with mAb 15308 (or would not be within the detection limits of microscopy with this antibody).

Alternatively, it is possible that the tags provided by the expression vectors could interfere with sequence-specific localisation signals for LBP/p40. Initially a role for cell cycle dependent factors in the ability of exogenous LBP/p40 to match mAb 15308 binding was questioned as preliminary staining attempts were performed soon after transient transfection. Whilst staining of stable transfectants at a later date argued against this, it is possible that expression of endogenous LBP/p40 is only switched on and processed transiently during the cell-cycle, whereas only a fraction of the exogenous protein, through being expressed continuously, receives the processing required for recognition by mAb 15308.

Uncertainty regarding the staining patterns of LBP/p40 can also be found in published literature regarding this protein. Although perfomed on tissue sections, observations that "Different antibodies (to LBP/p40) produce different staining patterns"have been made (Prof. S. Ghosh, personal communication) (Shmakov, Bode et al. 2000). Whereas the localisation has been stated as diffuse or vesicular, reports of cytoskeletal association (Keppel and Schaller 1991) are in keeping with the observed pattern of staining with mAb 15308 in these studies. The possible relationship between documented LBP/p40 biology and the epitope recognised by mAb 15308 is summarised schematically in figure 8-1.

When detecting LBP/p40 in pCDNA3.1D/V5-His-TOPO© using an antibody to the V5 tag, diffuse staining throughout the cytoplasm was seen. Conversely, LBP/p40 expressed as an EGFP fusion protein to the N-Terminus, localised mainly to the perinuclear region, nucleus and to a lesser amount in the cytoplasm. Again, these discrepancies could be due to either differing sensitivity of detection of the two tags or differing effects on intracellular localisation (C-terminal V5 vs N-terminal EGFP).

In any case, the inconsistency in location with these two forms of LBP/p40 highlights the fact that exogenous expression may not always provide a faithful reflection of a protein's physiological behaviour within a cell. Finally, it remains possible that the tags are cleaved at different stages during the processing of the protein resulting in a loss in detection of LBP/p40.

The finding that exogenously-expressed LBP/p40 as an EGFP fusion protein localised mainly to the nucleus and peri-plasmic regions (section 7.2.4) is also in keeping with some published reports (Sato, Kinoshita et al. 1996). The studies conducted by this group using epitope-tagged LBP/p40 led to the finding that the nuclear localisation of LBP/p40 was found to be mediated through binding to histones H2A, H2B, and H4 but not H3 (Sato, Kinoshita et al. 1996; Kinoshita, Kaneda et al. 1998). Future work aimed at clarifying the issues raised following these staining studies will be discussed later (section 8.2.4).

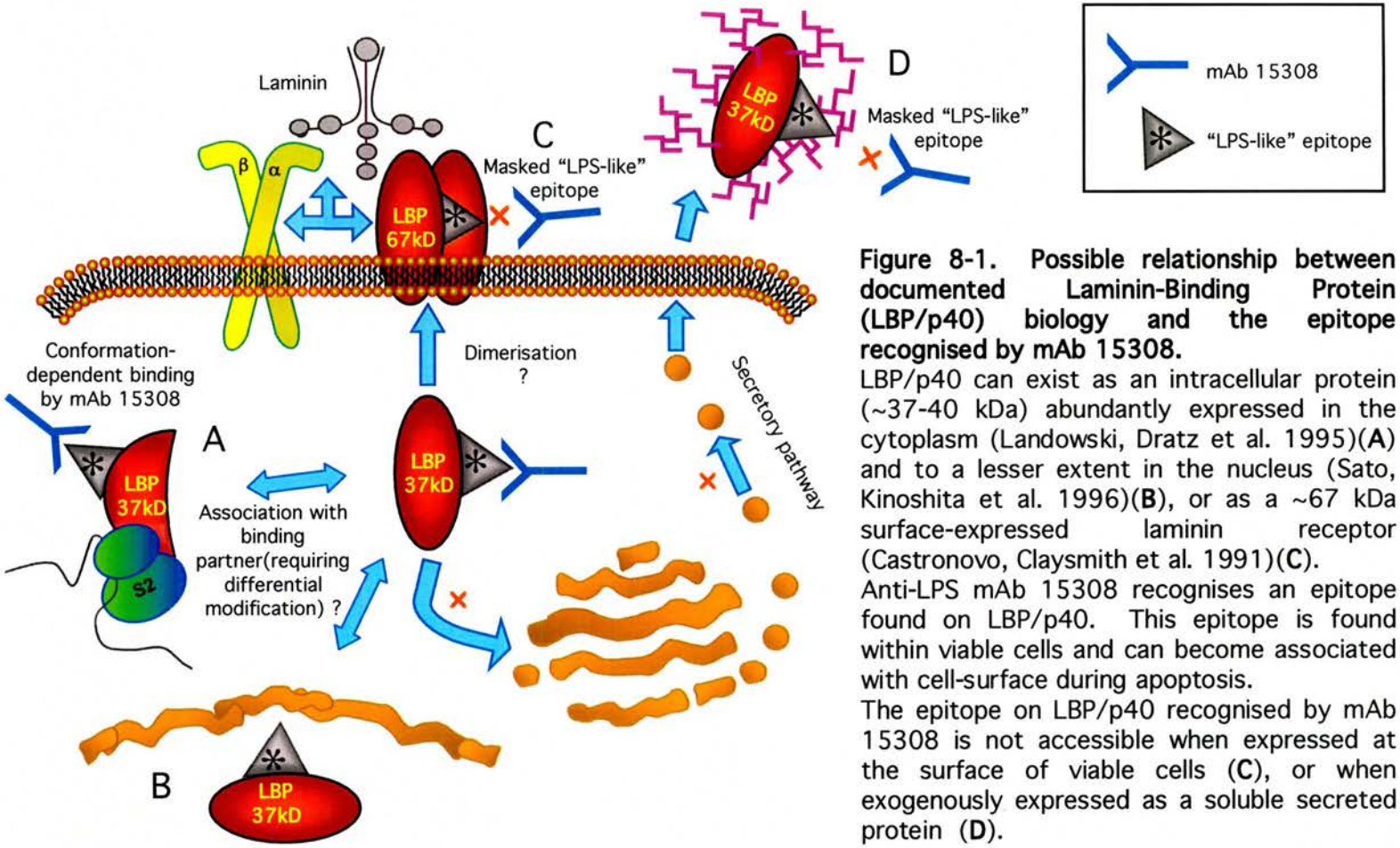


Figure 8-1. Possible relationship between documented Laminin-Binding Protein (LBP/p40) biology and the epitope recognised by mAb 15308.

LBP/p40 can exist as an intracellular protein (~37-40 kDa) abundantly expressed in the cytoplasm (Landowski, Dratz et al. 1995)(**A**) and to a lesser extent in the nucleus (Sato, Kinoshita et al. 1996)(**B**), or as a ~67 kDa surface-expressed laminin receptor (Castronovo, Claysmith et al. 1991)(**C**).

Anti-LPS mAb 15308 recognises an epitope found on LBP/p40. This epitope is found within viable cells and can become associated with cell-surface during apoptosis.

The epitope on LBP/p40 recognised by mAb 15308 is not accessible when expressed at the surface of viable cells (**C**), or when exogenously expressed as a soluble secreted protein (**D**).

8.2.3 Functional studies involving LBP/p40

Based on the potential role for an apoptotic-cell associated molecule bearing an "LPS-like" epitope in recognition by phagocytes several studies involving LBP/p40 were intended. However, due to time limitations and technical difficulties encountered whilst obtaining a stable endotoxin-free purified preparation of this protein at the time of writing this thesis, these studies were not completed. Thus, the following discussion summarises the results obtained at the time of documenting this work, whereas proposed further studies will be mentioned in later sections.

To ascertain whether LBP/p40 can interact directly with CD14, binding studies were performed using purified preparations of LBP/p40 and CD14-expressing cells. The nickel-metal affinity-purified preparations of rLBP/p40 derived from a bacterial expression system bound preferentially to CD14-expressing K562 cells compared to CD14-negative K562 cells (section 7.2.7). Given that this preparation contained endotoxin it is possible that LPS is responsible for this result. The removal of LPS from this preparation also resulted in loss of protein, making the concomitant loss in binding to CD14-expressing cells difficult to interpret (section 7.2.7).

The nickel-metal affinity-purified preparations derived from culture supernatants of transfected K562 cells did not retain the epitope recognised by mAb 15308, and adequate yields were not obtained from lysates of LBP/p40 transfected MCF-7 cells. Nevertheless, when binding experiments were carried using the K562-derived preparation in the presence of purified LPS, binding of these preparations to CD14 expressing cells was not observed (section 7.2.7), suggesting that a simple mixture of LBP/p40 and LPS is not sufficient to produce the result seen with the bacterial-cell derived preparation.

Production of sufficient quantities of functional LBP/p40 using an endotoxin-free insect system attempted to answer these issues conclusively. Preliminary tests using this preparation of LBP/p40 failed to demonstrate a preferential affinity for CD14-expressing K562 cells compared to CD14-negative K562 cells.

Nevertheless, as it is known that binding of LPS to CD14 is facilitated by LPS-binding proteins (Tobias, Mathison et al. 1988), and that bacteria themselves possess LPS-binding proteins (Hirschfeld, Ma et al. 2000), there is a possibility that the result obtained with the bacterial-derived preparation could be recreated through the addition of such transfer molecules to the LPS-free insect cell-derived preparation. However, time limitations prevented performance of an in-depth investigation of the role for LPS-binding proteins in enhancing interaction between LBP/p40 and CD14 or other PRRs.

8.2.4 Consolidating the findings described in this thesis

The results documented in this thesis provide scope for broadening studies in the area of markers associated with apoptotic cells. However, there are a number of important issues surrounding the foundational work performed thus far which require further investigation. These are summarised below: -

(1) Do "LPS-like" molecules on apoptotic cells play a role in their phagocytosis by macrophages? Answering this question would require a more rigorous testing of anti-LPS antibodies in recognition assays than those performed in section 6.2.3, perhaps testing cocktails of cell-reactive antibodies to investigate the possible confounding factor of multiple ligands for individual PRRs, or using Fab fragments to prevent the possible contribution of Fc-mediated phagocytosis.

(2) Of priority with respect to the reactivity of mAb 15308 will be to determine whether this antibody binds solely to LBP/p40 in its native form within cells, and if so, is specific posttranslational modification required? This could be addressed in a number of ways: -

- Immuno-depletion of endogenous LBP/p40 under native conditions within cell-lysates using the commercially-available antibody to LBP/p40 (Lam-R) followed by probing of the Lam-R negative (and hence LBP/p40 negative) lysates with mAb 15308.
- An alternative and perhaps more conclusive way to remove LBP/p40 would be to silence it's expression by RNA interference. This would allow a simple test for ablation of mAb 15308-reactivity to be assessed by immunofluorescence. Preliminary attempts to this effect have been made and the results are shown in appendix A8-1. However, Western blot analysis with Lam-R demonstrated only a modest reduction in protein levels following transfection with LBP/p40 siRNA suggesting a requirement for methodological optimisation. In the event that stable knock-down of LBP/p40 expression proves troublesome focus could be placed on sourcing or engineering LBP/p40 knock-out mice.
- Carrying out a more extensive testing of different cells lines stably expressing LBP/p40, or by microscopic analysis of LBP/p40 infected insect cells, as these expressed high levels of mAb 15308-reactive protein.
- *In vitro* translation of LBP/p40 could be used to determine the need for such posttranslational modification.

Answering these questions would also help clarify whether LBP/p40 is the molecule recognised by mAb 15308 at the surface of apoptotic cells prior to loss of membrane integrity.

In the following sections the discussion will focus on the structural basis for cross-reactivity between anti-LPS antibodies and apoptotic cells, centring on the existing molecular knowledge of LPS. Some suggestions for such investigations will be discussed with specifically with respect to LPS, but the same lines of investigation could also be applied to other ligands for PRRs such as peptidoglycan, lipoproteins and lipoteichoic acids.

8.3 Structural basis for interactions between anti-LPS antibodies and apoptotic-cell-associated epitopes

Much could be gained from understanding the observed capacity of anti-LPS antibodies to recognise apoptotic-cell-associated epitopes in terms of the exact molecules (or three-dimensional patterns of charge) conserved between the foreign and self structures which are directly involved in contacts with the immunoglobulin's binding-site. Knowledge of the precise epitope to which antibodies bind on ligands of PRRs such as CD14 could potentially be used to develop compounds capable of blocking LPS responsiveness by masking CD14 or could lead to a fuller understanding of how apoptotic cells exert their non-inflammatory/anti-inflammatory effects. However, at present it is unknown whether LPS binds directly to CD14 or if a bridging molecule is required (da Silva Correia, Soldau et al. 2001). Why apoptotic cells do not signal through TLRs despite being recognised by the same phagocytic PRRs associated with TLR-activity in response to microbes is unknown (Blander and Medzhitov 2004). Nevertheless, one can make two broad proposals to explain the cross-reactivity observed during this work:- (1) either matching chemical structures to parts of LPS exist on molecules inside eukaryotic cells, or (2) the molecules inside eukaryotic cells simply bear a similar pattern of electrostatic charge/hydrophobicity to LPS enabling their recognition by anti-LPS Abs. In the former scenario the shared structures may be of carbohydrate, lipid or phosphate origin. One can suggest conditions for each based on what is known about CD14 ligands and cross-reactivity of LPS Abs. These are discussed below.

The precise chemical groups on LPS to which the broadly cross-reactive antibodies used in this work bind have not been determined. Common core carbohydrate

determinants are assumed to be the targets as the O-antigen of LPS is structurally hypervariable. Evidence supporting this presumption is supplied by a study into the isotypes of a panel of cross-reactive mAbs performed by Pollack *et al.* They found all IgGs (16 of 29) recognising core sugars were of the 2a or 3 isotype with no type 1, whereas those to lipid A (13 of 29) were all of IgM class (Pollack, Chia et al. 1989). Alternatively, one can draw structural similarities between the repeating units of hexoses or N-acetyl linked hexoses found in O-antigens and mammalian glycoconjugates. Epitope mapping would provide conclusive insight into the structural requirement for the binding of these antibodies to LPS and crystallographic data could be utilised to reveal structural similarity between the LPS and the proposed ACAMP.

To investigate whether conserved carbohydrate structures are indeed responsible for the antibody cross-reactivity described in this thesis one may refer to the techniques employed by their manufacturers to characterise their microbial specificity. Thus, in addition to carrying out ELISAs on purified LPS, the suppliers of the anti-LPS Abs used in this work characterised their LPS specificity by testing binding to whole bacterial cells that had been treated with Proteinase K to strip the cell surfaces of protein moieties, and periodate to oxidise carbohydrate moieties (such as those of LPS). Reactivity of all anti-LPS antibodies was unaffected by proteinase K, yet periodate treatment resulted in loss of reactivity with whole cells (Personal communication, Tom Glickman, QED Biosciences). Repeating these studies using apoptotic cells may provide an insight into the nature of the epitope recognised, although it must be appreciated that not all protein epitopes are sensitive to

Proteinase K and that periodate can oxidise many more structures than carbohydrates.

Many reliable techniques exist to study carbohydrate-containing molecules. Lectins can be used specifically to probe for the presence of mono- and oligosaccharides. Indeed, preliminary studies using a panel of 14 lectins, screened for differential binding to apoptotic or viable Mutu I cells, identified Dolichos biflorus (DBA), a lectin that binds specifically to N-Acetyl-galactosamine, as having a preference for dead cells (Ceri Oldreive - personal communication). Subsequent studies revealed that this lectin, as was observed for the anti-LPS antibodies, bound to late apoptotic cells and permeabilised viable cells. Also of interest is the fact that N-Acetyl-galactosamine residues can also be found in the core regions of some LPSs (Yokota, Ohtsuka et al. 1996; Vinogradov and Sidorczyk 2001) and that N-Acetyl-linked sugar residues are a form of post-translational modification for nuclear and cytoplasmic proteins found in mammals (Zachara, Cheung et al. 2004). In considering these facts, it would be of interest to establish whether the observed apoptotic-cell-reactivity is due to the presence of N-Acetyl-galactosamine groups on apoptotic-cell-associated epitopes by looking for competition for staining between antibodies and DBA. Another possibility would be to examine the effects of treating cells with panels of glycosidases or inhibitors of glycoprotein processing, on antibody binding.

It is also possible to draw structural similarities between ceramide (Joseph, Wright et al. 1994) or phosphatidyl inositides and lipid A of LPS (all three being CD14 ligands) (Wang, Kitchens et al. 1998). For example, the long fatty acid carbon

chains of ceramide and LPS show identical conformational placement on the backbones of each molecule (Joseph, Wright et al. 1994).

To investigate whether conserved lipid structures are required for the recognition of ACAMPs by the anti-LPS mAbs one could apply simple lipid overlay tests using lysates of mammalian cells and compare probed samples to purified preparations of known lipids (Cowart, Szulc et al. 2002). This approach could also be used to examine the requirements for apoptotic-cell-binding by mAbs 15306, 15174 (and other potential apoptotic-cell binding anti-LPS mAbs) in addition to testing the effects of various fixation and permeabilisation methods, to investigate the observed basis for sensitivity of mAb 15174 to IntraStain (DAKO) (c.f. section 6.2).

The involvement of structural mimicry in dual recognition has previously been examined following the detection of antibodies to lipid-A in the synovial fluid and serum from children with oligoarticular arthritis. Reactivity of these antibodies towards lipid-A can be inhibited by collagen types I and II, cardiolipin, and denatured DNA (Miller, Zhu et al. 1996). In this instance, antibody recognition was found to be dependent on contacts with diphosphate groups of lipid A. Thus, phosphorylated protein moieties could well be responsible for apoptotic-cell binding. Figure 8-2 summarises potential structures associated with intracellular eukaryotic molecules which anti-LPS antibodies could recognise.

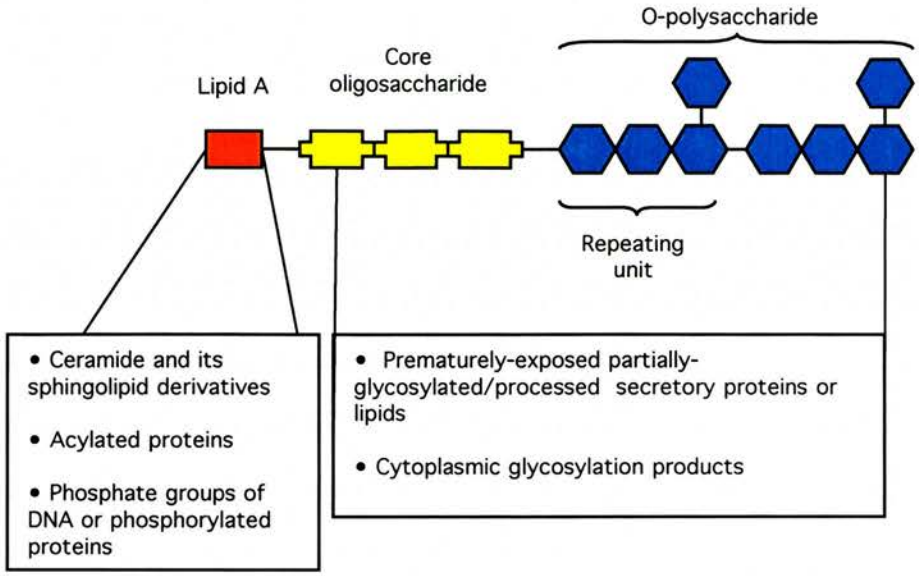


Figure 8-2. Potential structures associated with intracellular eukaryotic molecules which anti-LPS antibodies could recognise. Several possibilities exist for matching chemical structures found on molecules inside eukaryotic cells to parts of LPS. The shared structures may be of carbohydrate, lipid or phosphate origin. However it is also appreciated that cross-reactivity of anti-LPS antibodies might be mediated via a similar patterns of ionic charge or hydrophobicity rather than matching chemical groups.

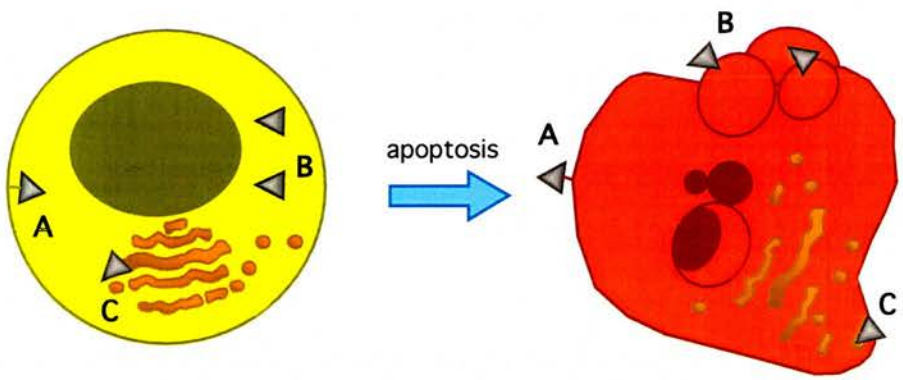


Figure 8-3. Possible routes by which ACAMPs might become exposed during apoptosis. ACAMPs appear on the surface of dying-self as a result of losing membrane asymmetry (Fadok, Voelker et al. 1992)(**A**), translocation of cytoplasmic compartments (Arur, Uche et al. 2003)(**B**), or the premature exposure of endoplasmic reticulum/ Golgi components at the cell-surface (Morris, Hargreaves et al. 1984; Lane, Lucocq et al. 2002) (**C**).

8.3.1 Structural basis for recognition of LBP/p40 by mAb 15308

The increase in molecular weight resulting from expression of LBP/p40 following transport through the secretory pathway in K562 cells (section 7.2.3.4) may in part have been due to glycosylation as LBP/p40 has a number of potential sites for O-linked glycosylation (although none for N-linked glycosylation). Endogenous LBP/p40 does not possess a classical leader sequence for transport through the secretory pathway (Yow, Wong et al. 1988). Recognition of LBP/p40 by mAb 15308 and a commercial anti-LBP/p40 antibody was lost following expression via this route, which suggests that the modifications imparted by processing through the secretory pathway resulted in loss of (or masking of) the epitope required. This could also be interpreted as evidence that O-linked sugar chains do not provide the means by which anti-LPS mAb 15308 can bind LBP/p40. This does not exclude the possiblilty that other less well-known forms of cytoplasmic glycosylation, such as the addition of N-Acetyl-linked sugar residues to serine and threonines (Zachara, Cheung et al. 2004) could provide suitable carbohydrate residues for recognition by an anti-LPS antibody. However, the reagents required to examine such modifications are not widely available.

In considering other forms of post-translational modification which could provide chemical groups for recognition by an anti-LPS mAb, reports that LBP/p40 can act as a substrate for the cell-cycle-specific kinase CDC2 (Keppel, Fenger et al. 1997) and that the protein is acylated (Landowski, Dratz et al. 1995) would suggest phosphate groups or lipids as a possible basis for recognition. Whilst structural predictions could be made based upon the reported reactivity of mAb 15308 towards LPSs from different strains of *E. coli,* cross-reactivity could simply be due to a

certain configuration of amino-acid residues on LBP/p40 that resembles LPSs through a similar three-dimensional pattern of ionic charge.

The biological implications of epitope conservation are discussed in the following sections.

8.4 Implications for LPS-like molecular structure for the biology of apoptosis, inflammation and immunity

8.4.1 Involvement of epitopes recognised by anti-LPS mAbs in the recognition of apoptotic cells

The observation that "LPS-like" epitopes are displayed on the surface of apoptotic cells raises the question of whether they may function in the recognition and clearance of apoptotic remains. Although initial studies have failed to demonstrate blocking of apoptotic-cell interaction using the apoptotic-cell cross-reactive anti-LPS Abs (section 6.2.3) it is possible that blocking a single "LPS-like" pattern amongst the many different patterns within or on an apoptotic cell that PRRs such as CD14 can recognise, may be insufficient to compromise apoptotic-cell clearance. The possibility of multiple distinct pathogen-like epitopes mediating interaction within an apoptotic-cell phagocyte synapse could be investigated by testing a cocktail of similar antibodies that demonstrate reactivity towards apoptotic cells. Such a panel could be formed from antibodies to pathogen-associated ligands in addition to LPS and of PRRs other than CD14.

Besides CD14, the apoptotic-cell opsonins, SP-A, SP-D, MBL and C1q, have also been shown to bind core regions of LPS (Zohair, Chesne et al. 1989; Van Iwaarden, Pikaar et al. 1994; Devyatyarova-Johnson, Rees et al. 2000). The findings of this

thesis provide fuel for the hypothesis that apoptotic-cell-associated "LPS-like" epitopes form the ligands that allow these innate receptors to participate in the engulfment of dying self. To investigate whether PRRs such as SP-A, SP-D, and C1q use the same contact surfaces to bind apoptotic cells as they do to bind LPS, the ability of LPS and anti-LPS Abs to compete with these opsonins for binding to apoptotic cells could be assessed. It would also be of interest to know whether the epitopes recognised by each of these receptors are exposed in unison or if there is a hierarchy in their appearance. Regardless of the timing, once at the surface, these LPS-like epitopes also have implications for understanding tolerance and autoimmunity and the normal response to apoptotic cells.

8.4.2 Implications for immune responses

8.4.2.1 Immune response to apoptotic cells

It is tempting to speculate how the property of dual recognition of apoptotic cells and pathogens by PRRs like CD14 might reflect general features of autoimmunity. The ability of pathogen-specific T or B cells to interact with similar structures found on dying self increases the probability of raising an autoimmune response in situations where dying cells and pathogens are encountered in close proximity. In other words this could explain how immune effector cells designed to see pathogen-derived epitopes could cross-react with self-antigens displayed on dying cells to drive autoreactivity by molecular mimicry.

Finally, as mentioned previously, TLRs, although not implicated in apoptotic-cell clearance can be stimulated by endogenous ligands. For example, whilst normally inactive or inhibitory to TLR9 signalling, mammalian DNA can stimulate this

receptor when present in immune complexes (Leadbetter, Rifkin et al. 2002). Therefore it is not unreasonable to imagine the potential for immune complexes of "LPS-like" structures similarly to trigger TLR dependent signalling pathways.

8.4.2.2 Immune response to pathogens

In considering the findings of this thesis one may also expect that an excess of apoptotic cells could lead to an impaired ability to fight infection. Incidents of this kind have been observed in other disease settings as it has been shown that dextran-binding antibodies from plasma of human donors bind to glycoconjugates from yeast and to lipopolysaccharides from Klebsiella and group A Streptococci. Inhibition by galactose and glucose as well as certain disaccharides was obtained at concentrations below those reached in diabetic sera suggesting a reason for the increased susceptibility of diabetics to infection (Chacko and Appukuttan 2003).

Taking a step further back, cross-reactivity of this kind raises the question of how B cells specific for these molecules escape from negative selection, as these structures could well be presented on the surface of dying cells in the germinal centres. Thus, the exposure of "LPS-like" structures on the surface of apoptotic blebs would suggest tolerance to LPS in the periphery as interactions between immature and B cells and surface-exposed LPS-like structures on a neighbouring apoptotic cell could trigger strong stimuli toward central deletion. However, the moieties expressed by apoptotic cells are unlikely to represent entire LPS molecules allowing those B-cells specific for the remaining portions of LPS to mount an immune response. On the other hand, as the surface exposure was seen to be a late phenomenon, clearance mechanisms may exist in areas of B-cell development which remove the dying cells before the exposure of such epitopes at these sites, leaving the exposure of these

patterns to occur on "late" apoptotic cells under other circumstances in the periphery where different recognition systems may operate.

8.4.2.3 Potential for modulating the normal response to apoptotic cells

Although structural conservation between LPS and apopotic-cell-associated epitopes has been demonstrated in this work, one would expect that functional mimicry does not necessarily occur as LPS is proinflammatory whereas apoptotic cells are believed to be anti-inflammatory (Fadok, Bratton et al. 1998). It is possible that apoptotic cells may provide an overriding signal to inhibit stimuli provided by "LPS-like" structures or it could be that apoptotic cells only display structures resembling the non-toxic portions of endotoxin. For example, it has been possible to demonstrate that, by comparing certain structural analogues of lipid-A, to that of low-toxicity LPS (from certain bacterial strains) the supramolecular requirements for LPS bioactivity correlate with the type of aggregates the molecule allows itself to form (Labischinski, Barnickel et al. 1985; Golenbock, Hampton et al. 1991). That is, Lipid A samples which adopt a cylindrical conformation are biologically inactive, whereas those which form a conical conformation are highly active (Schromm, Brandenburg et al. 1998). This knowledge may in the future provide a means to subvert the immune system against apoptotic cells, for beneficial therapeutic ends, for example, switching environments that have high rates of apoptosis (such as certain solid tumours) into an inflammatory setting.

It has already been shown *in vitro* that opsonisation of apoptotic cells with immunoglobulin can result in both a failure to induce the TGF-β production, usually

observed from macrophages during programmed cell clearance, and a concomitant rise in TNF-α production (Fadok, Bratton et al. 2001; Huynh, Fadok et al. 2002). Indeed, initial attempts to recreate these results by opsonising apoptotic cells with the panel of anti-LPS antibodies used in this thesis produced a similar effect. In the event that LBP/p40 proves able to function as a ligand for CD14 (or another PRR) there is also potential to use this molecule as a target for therapy.

8.4.3 Working model

In considering the results of this work it is tempting to speculate that multi-cellular organisms have evolved to "hide" certain groups of epitopes (intracellularly) from the immune system (as opposed to the immune system simply looking for non-self). What factors could determine whether a particular pattern can be exposed to the extracellular milieu? Such segregation could be on the basis of overall electrostatic charge or hydrophobicity, similar to the "hyppos" described by Polly Matzinger and colleagues (Matzinger 2002). By this definition such molecules could be either microbial, non-non-microbial, derived from intracellular host compartments or sites inaccessible to the immune system under normal circumstances. This concept is in keeping with existing evidence of how ACAMPs appear on the surface of dying-self, such as exposure by loss of membrane asymmetry (Fadok, Voelker et al. 1992), translocation of cytoplasmic compartments (Arur, Uche et al. 2003), or the premature exposure of endoplasmic reticulum/ Golgi components at the cell-surface (Morris, Hargreaves et al. 1984; Lane, Lucocq et al. 2002), as summarised in figure 8-3.

8.5 Broadening the understanding of these findings

The central theme to this thesis, that apoptotic cells can expose epitopes bearing structural resemblance to PAMPs, has been formed from studying the characteristics of a relatively limited panel of antibodies. There is therefore great potential to extend these studies further and to deepen the understanding of the observations made thus far.

8.5.1 Investigating the potential for other LPS-binding proteins to recognise apoptotic cells

In addition to screening new anti-LPS antibodies for apoptotic-cell binding capacity, there is potential to return to the investigation of innate LPS-binding proteins as much information exists regarding the location of amino acids involved in their interaction with LPS. These molecules include "antimicrobial peptides"such as the α-defensins, lipid-binding proteins found in serum such as phospholipids-transfer protein, opsonins such as serum amyloid P, and membrane bound molecules involved in signalling such as MD-2 (reviewed in Chaby 2004). Interestingly LPS has been shown to bind to histones, further supporting the notion that LBP/p40 possesses an "LPS-like" epitope, as it too associates with histones (Augusto, Decottignies et al. 2003). Thus, there is potential for testing the common structural motifs required by these molecules for their association with LPS for use as diagnostics or therapeutics in area of apoptosis research.

Finally, a number of LPS-binding proteins from prokaryotic organisms could be screened for their reactivity towards dying eukaryotic cells. An example of

preliminary attempts to explore the possibility that eukaryote-derived LPS-binding proteins interact with apoptotic cells is detailed in the following section.

8.5.1.1 The T4 bacteriophage "docking system" and LPS binding

One well-characterised LPS-binding protein is that which forms part of the bacteriophage T4 "docking system". In order for T4 phage to bind to their host bacterium, firstly long tail fibres recognise outer membrane proteins before short tail fibres extend and bind irreversibly to the core-region of LPS (Riede 1987). The crystal structure of this LPS-binding-protein with its bound lignad has been solved (Thomassen, Gielen et al. 2003), and a recombinant form employed as an LPS decontamination system (see http://www.profos.de/technology.php). As part of preliminary studies aimed at extending our understanding of "LPS-like" structures on apoptotic cells, T4 phage were obtained with the intention of testing their ability to bind apoptotic cells in preference to viable cells. The phage particles were supplied freeze-dried in skimmed-milk powder. This preparation was biotinylated and tested for the ability to bind apoptotic cells over and above a solution of biotinylated skimmed milk (to represent a "carrier control"). Both preparations bound to apoptotic cells. On contemplation, the viability of this result was quite apparent given the numerous innate immune components present in milk. Indeed, subsequent studies have revealed lactoferrin to be the major active component in milk responsible for apoptotic cell binding (Andrew Devitt, John Pound and Chris Gregory, unpublished) and lactoferrin itself is known to be an LPS-binding protein (Na, Han et al. 2004).

8.5.2 Rationalising flaws in the phage antibody approach to identifying ACAMPs

Attempts to generate apoptotic-cell-binding phage antibodies resulted in the isolation of cell-binding monoclonal phage antibodies with a much lower affinity for populations of apoptosis-induced cells than viable populations (section 3).

In section 5.6 it was hypothesised that epitopes resembling PAMPs found within the bacterial cultures used to propagate bacteriophage libraries might have resulted in the loss of phage clones displaying antibody fragments with high affinity for ACAMPs. The finding that lactoferrin from milk binds to apoptotic cells may have revealed an additional fatal flaw in the way that these early experiments were performed as milk powder was used for blocking both the phage library and the apoptotic cells during selection. Thus it is possible that novel ACAMPs were being masked by innate receptors present in the milk solution.

8.5.3 Concluding remarks

The view established in this thesis, that conserved molecular patterns exist associated with both dying-self and microbes, sets a new paradigm for studying markers of apoptosis. Whilst a functional role for "LPS-like" structures on apoptotic cells needs to be evaluated further, studies using this approach might help in understanding the progression of inflammatory, infectious and tumoural processes. Many aspects of this model such as the possible conservation of conserved microbial structures other than "LPS-like" moieties are readily open for investigation and may soon allow the expansion of our knowledge of phagocyte interaction with apoptotic cells.

Buffers and Solutions

0.5M EDTA pH8.0

For 500ml	EDTA	93.05g
	dH_2O	400ml
Adjust to pH8.0 with conc. NaOH		
Adjust to final volume with dH_2O		

5xTBE

For 5000 ml	Tris base	270g
	Boric acid	137.5g
	0.5M EDTA	50ml
Adjust to final volume with dH_2O		

1M Tris HCl

For 1000ml	Tris base	121.1g
	dH_2O	800ml
Adjust to desired pH with conc. HCl		
Adjust to final volume with dH_2O		

1xTE

For 500mls	1M Tris-HCl pH8.0	5ml
	0.5M EDTA	1ml
Adjust to final volume with dH_2O		

1% agarose gel

	Add agarose (1% w/v) to 1xTBE	
Heat until agarose goes into solution		

6xDNA loading buffer

	Bromophenol blue	0.25%
	Xylene cyanol FF	0.25%
	Glycerol	30%
Adjust to final volume with dH_2O		

10% Resolving gel

	30% acylimide	3.3ml
	10%SDS	0.1ml
	1M Tris pH8.8	2.5ml
	10% APS	0.15ml
	TEMED	5µl
	dH_2O	4ml

4% Stacking gel

	30% acylimide	1.3ml
	10%SDS	0.1ml
	1M Tris pH6.8	2.5ml
	10% APS	0.15ml
	TEMED	10µl
	dH_2O	6.1ml

10xTOWBIN buffer

For 2000ml	Tris base	60.6g
	Glycine	288.4g
Adjust to final volume with dH_2O		

Laemli sample buffer

For 8ml	0.5M Tris-HCl	1ml
	10% w/v SDS	1.6ml
	Glycerol	0.8ml
	2-Meracptoethanol	0.4ml
	1% w/v Bromophenol Blue	0.4ml
	dH_2O	3,8ml

SDS-PAGE Running buffer

For 1000ml	10xTOWBIN buffer	100ml
	10%SDS	10ml
Adjust to final volume with dH_2O		

GTE

For 1000ml	glucose	9g
	1M Tris at ph 8.0	25ml
	EDTA	20ml
Adjust to final volume with dH_2O		

Potassium acetate(3M K+/5M Ac-)

	5M potassium acetate	50ml
	glacial acetic acid	11.5ml
	distilled water	28.5ml

TE

For 1000ml	1M Tris at ph 8.0	10ml
	EDTA	2ml

1x Phosphated-Buffered Saline (PBS)

For 1000ml	sodium chloride	8.0g
	potassium chloride	0.2g
	potassium dihydrogen phosphate	0.2g
Adjust to pH7.4	disodium hydrogen phosphate	1.145g
Adjust to final volume with dH_2O		

1xPBS-T

For 1000mls	1xPBS	500ml
	Tween 20	0.5ml
Adjust to final volume with dH_2O		

5%MPBS

For 100mls	1xPBS	100ml
	Non-fat milk powder	5g

Tris-buffered Saline (TBS)

For 1000ml	sodium chloride	8.0g
	potassium chloride	0.2g
	Tris Base	3g
Adjust to pH7.4		
Adjust to final volume with dH_2O		

50 mM sodium phosphate buffer

For 1000ml	sodium chloride	17.4g
	sodium dihydrogen orthophosphate dihydrate	7.1g
Adjust to pH8.0		
Adjust to final volume with dH_2O		

LB

For 1 litre	Bacto tryptone	10g
	Bacto yeast extract	5g
	sodium chloride	10g
Adjust pH to 7.2 using 1M NaOH.	Add antibiotics as appropriate	
Adjust to final volume with dH_2O		

LB Agar

Add 15g of agar to 1 litre of LB	Add antibiotics as appropriate

NZY broth

For 1 litre	Bacto tryptone	10g
	Bacto yeast extract	5g
	sodium chloride	5g
Adjust pH to 7.2 using 1M NaOH.	Magnesium sulphate	2g
Adjust to final volume with dH_2O		

Annexin V binding buffer (AxVBB)

For 100ml	0.5 M HEPES , ph 7.4	2ml
	1.5M sodium chloride	9.3ml
	0.1M calcium chloride	2.5ml
Adjust to final volume with dH_2O		

3% Paraformaldeyhde

For 500ml	paraformaldehyde	15g
	1xPBS	500ml
	1M magnesium chloride	0.5ml
	1M calcium chloride	0.5ml

2 x Hepes buffered saline (HBS)

For 1000ml	sodium chloride	16g
	phosphate(dihydrate)	0.27g
	Hepes	10g
	dextrose	2g
Adjust pH to 7.05		
Adjust to final volume with dH_2O		

Percoll gradients

51%	90% v/v percoll in saline	1.02ml
	Platelet poor plasma	0.98ml
42%	90% v/v percoll in saline	0.84ml
	Platelet poor plasma	1.16ml

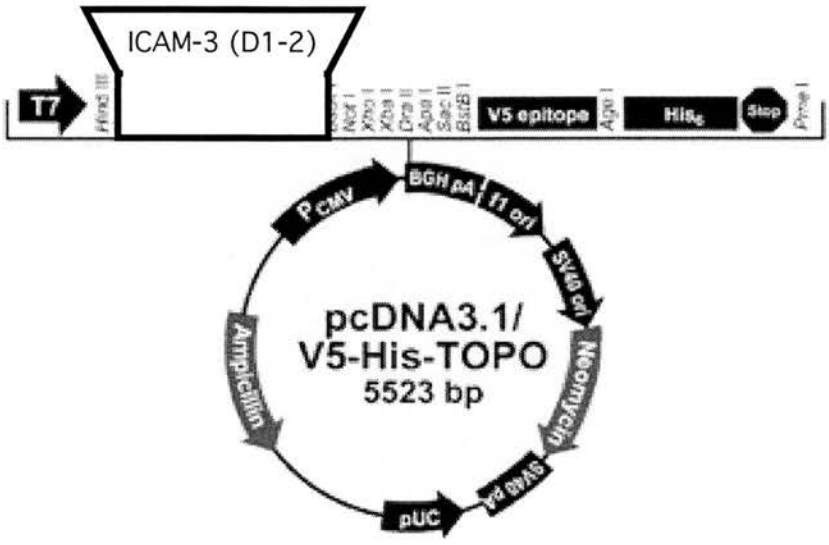
Macrophage assay medium

	bovine serum albumin(BSA)	0.2g
	RPMI	100ml

50 xJenner-Giemsa buffer

For 1000ml		
Solution A	orthophosphate dihydrate	6.24g
	dH_2O	200ml
Solution B	disodium hydrogen phosphate	2.84g
	dH_2O	100ml
5.6		
Adjust to final volume with dH_2O		

A



B

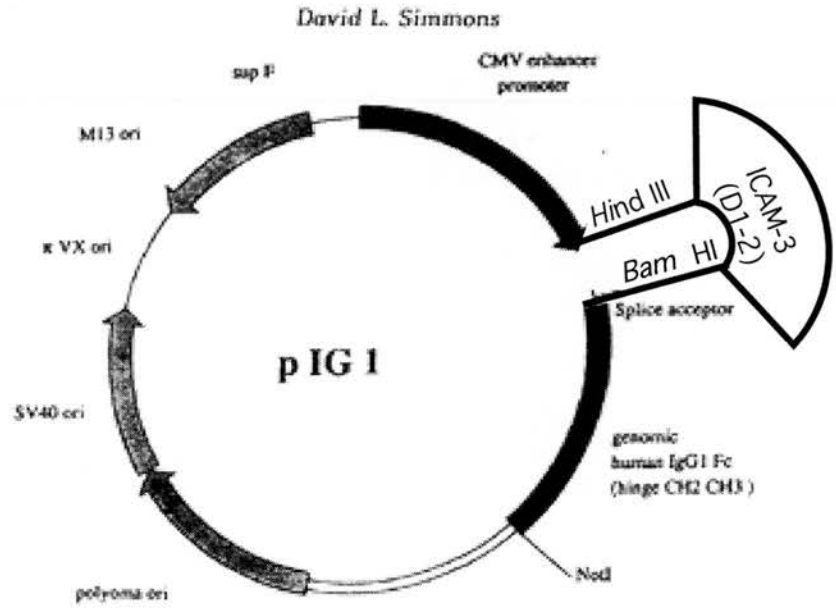


Figure A4-1 Maps of plasmid vectors used for expressing recombinant ICAM-3. A; Domains 1&2 (D1-2) of ICAM-3 cloned into pcDNA3.1/V5-His-TOPO for expression of histidine-tagged ICAM-3. B; ICAM-3 (D1-2) cloned into pIG 1 for expression of ICAM-3-Fc.

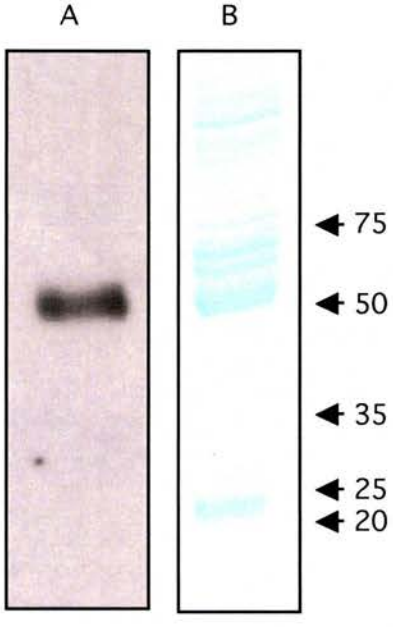


Figure A 4-2. Quality assessment of nickel-metal affinity purified ICAM-3-Fc. Polyhistidine-tagged ICAM-3(His-I3) was expressed as a secreted protein in 293^T cells. A; Western blot of purified His-I3. Preparations were analysed by probing with an antibody to the C-terminal V5 tag. B; The presence of proteins other than His-I3 was assessed by examination of Coomassie-blue-stained 10% SDS-polyacrylamide gels.

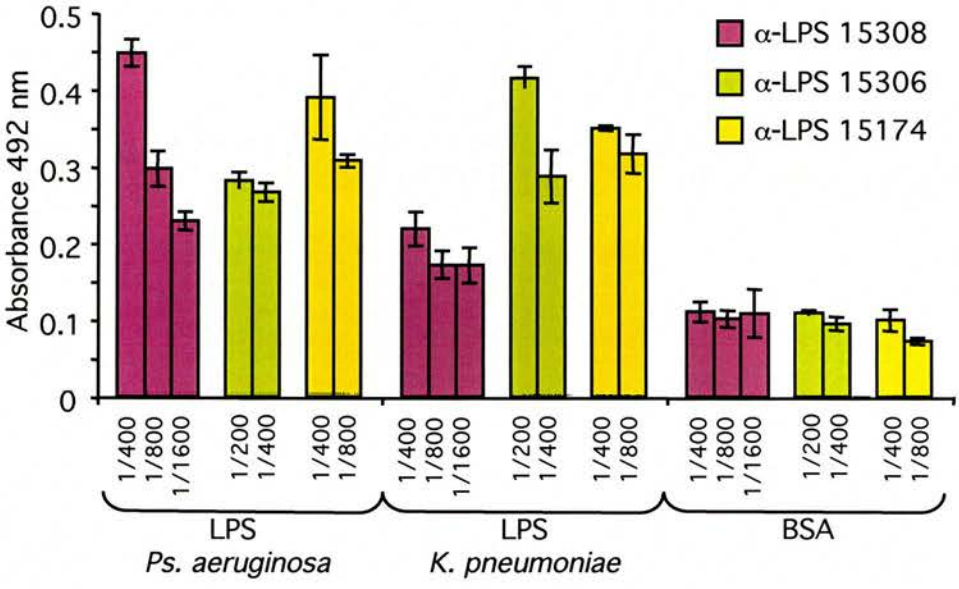
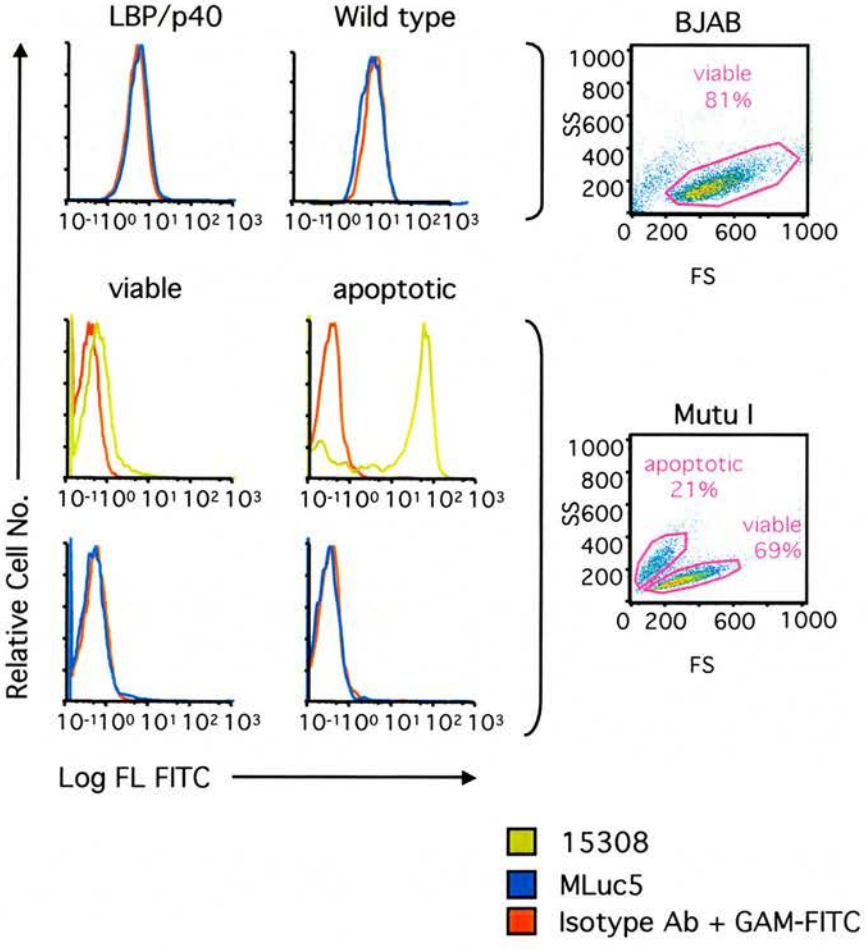


Figure A6-1. Binding of anti-LPS mAbs to purified LPS.
An ELISA against purified LPS from *Pseudomonas aeruginosa* (*Ps. aeruginosa*) and *Klebsiella pneumoniae* (*K. pneumoniae*) was used to confirm the reactivity patterns of mAb 15308. As stated by QED Biosciences Inc mAb 15308 bound preferentially to LPS from *Ps. aeruginosa*. However testing of mAb 15306 also showed reactivity towards purified LPS in this system despite not being documented as specific for these types of LPS by QED Biosciences (http://www.qedbio.com/alpha_e_g.htm). Indeed, all three α-LPS antibodies bound to both types of purified LPS at significantly higher levels than to BSA in a concentration-dependent manner. MAb 15174 that is actually marketed as a Chlamydia trachomatis mAb also displayed binding to these preparations. Isotype antibodies did not react with these LPS preparations above the observed level for BSA (data not shown).
Microtiter plates (Immulon II) were coated either with LPS purified from the indicated bacteria or with BSA overnight at RT.
The binding of the anti-LPS mAbs to coated wells was then assayed by ELISA (see methods section 2.11.1.1) at the dilutions indicated below each bar. Results shown are the mean +/- S.D. of duplicate wells. Representative of two independent assays.



Appendix A7-1. Example results of cell lines testing negative for expression of the 67kDa form of LBP/p40 as determined by staining with mAb MLuc5. The binding of mAb MLuc5 (anti-67kDa LBP/p40) to viable BJAB (either wild type or LBP/p40 transfected) cells or Mutu I cells treated with ionomycin for 16 hours was assessed by flow cytometry. Bound antibody was detected with goat anti-mouse secondary antibody labeled with FITC. Staining of apoptotic cells with mAb 15308 was included as a positive control. Within the Mutu I population, cells were identified as viable or apoptotic by their light scattering properties (see methods section 2.3.3.3).

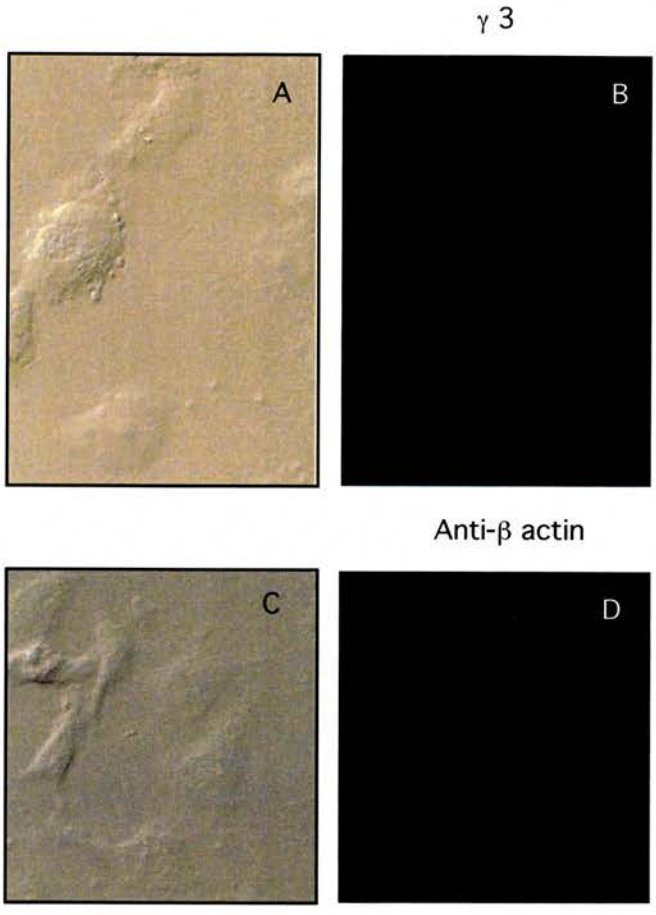


Figure A7-2. Neither anti-β-actin nor an isotype-matched antibody to 15308 bind to the surface of apoptotic MCF-7 cells, as determined by microscopy.
MCF-7 cells were treated with etoposide for 48-72 hours to undergo apoptosis. Binding of the anti-β-actin antibody (clone AC15) and isotype antibody to mAb 15308 (γ3) was detected with goat anti-mouse secondary antibody labeled with AlexaFluor-488 (green). In contrast to mAb 15308 (section 7.2.6) no staining of surface blebs was observed.

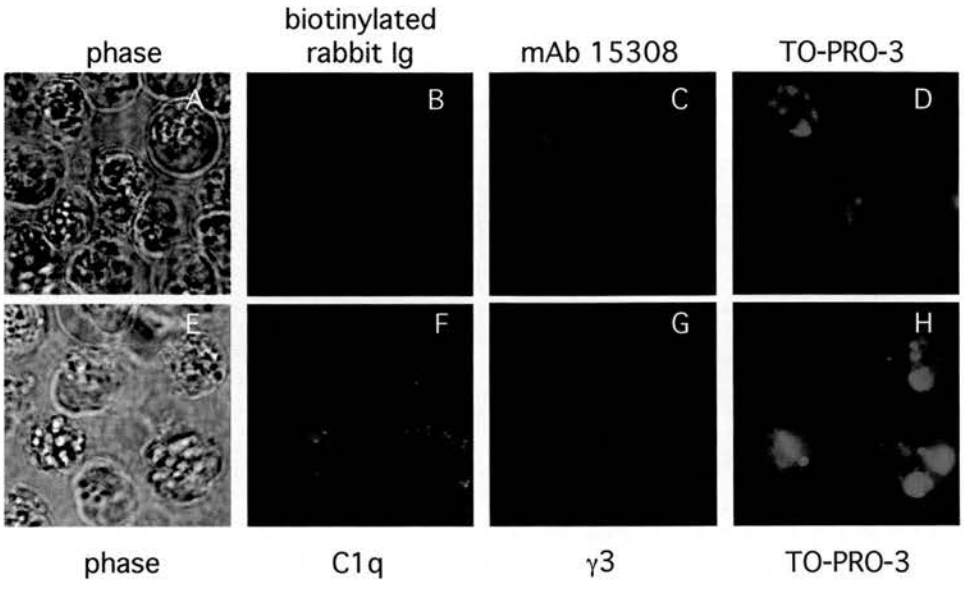


Figure A7-3. Control staining for studies investigating co-distribution of 15308 reactivity and C1q on apoptotic Mutu I cells. Mutu I cells were treated with ionomycin for 16 hours to undergo apoptosis, and the epitopes recognised by mAb 15308 and C1q examined by confocal microscopy. Negative control staining with isotype-matched antibody to mAb 15308 (γ3, panel G) or biotinylated rabbit IgG (panel B) as a control protein for C1q was performed at the same concentrations. Mouse antibodies were visualised using goat anti-mouse secondary antibody labeled with AlexaFluor-568 (red), biotinylated proteins were visualised using streptavidin labeled with AlexaFluor-488 (green). Shown are the confocal fluorescence images of two separate fields of view. **A-D** ; images from a field of view examining control staining for C1q. **E-F** ; images from a field of view examining control staining for mAb 15308.

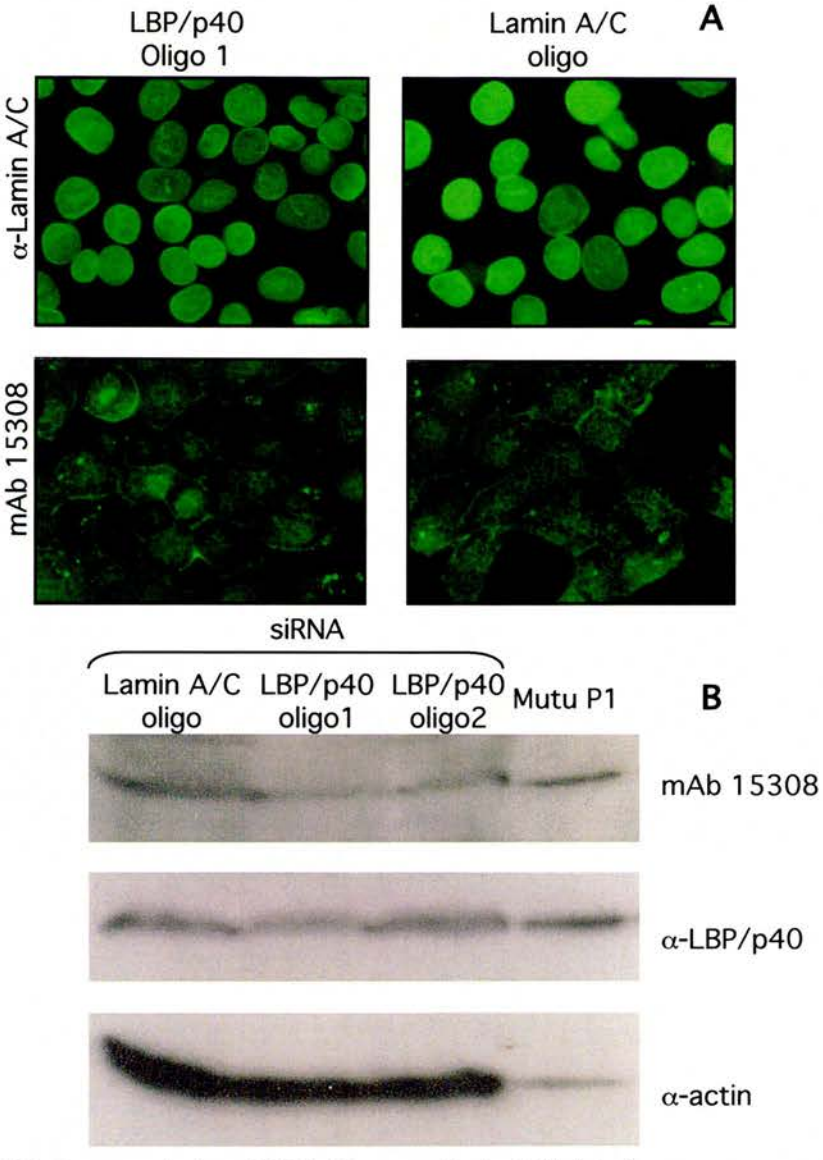


Figure A8-1 . Downregulation of LBP/p40 expression by RNA interference.
To confirm or refute the sole reactivity of mAb 15308 towards endogenously expressed LBP/p40 in its native form, attempts were made to downregulate LBP/p40 expression using siRNA. HeLa cells were transfected with two siRNA oligonucleotide duplexes (oligo) for LBP/p40. As a control, siRNA duplexes for Lamin A/C were included. **A;** Immunofluorescence analysis and assessment by microscopy with α-Lamin-A/C and mAb 15308 revealed few cells to have reduced fluorescence in LBP/p40 siRNA-transfected cells compared to control lamin A/C siRNA transfected cells. However little silencing of Lamin-A/C was seen in lamin A/C siRNA transfected cells compared to LBP/p40 siRNA transfected cell when probing with α-Lamin-A/C. **B;** Western blot analysis with both a commercially available antibody to LBP/p40 (Lam-R) and 15308 demonstrated only a modest reduction when using LBP/p40 siRNA duplexes used to transfect cells compared to control lamin A/C siRNA transfected cells. A very strong signal was observed when probing for β-actin as a loading control. As it is hard to gauge differences in such a strong signal, the small differences seen when probing for LBP/p40 cannot be taken as conclusive evidence of gene silencing. Taken together these results indicated that the silencing procedure was not successful. At the time of these studies attempts made to use stably knockdown LBP/p40 in order to circumvent interpretational problems associated with turnover of this protein.

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